

# Chronic Probiotic Supplementation and its Effects on eHsp72 and LPS Concentration Following a Desert-based Ultramarathon

Hannah Marshall

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Chronic Probiotic Supplementation and its Effects  
on eHsp72 and LPS Concentration Following a  
Desert-based Ultramarathon

By

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A thesis submitted to the University of Bedfordshire in partial  
fulfillment of the requirements for the degree of Masters of  
Science by Research


December 2015

## **Author's Declaration**

I declare that this thesis is entirely my own work. It is being submitted for the degree of Masters of Science by Research at the University of Bedfordshire. It has not been submitted before for any degree or examination in any other University.

This research was conducted in collaboration with the Sport and Exercise Science Research Group at Anglia Ruskin University, Cambridge.

I declare that the word count of this thesis is 17,392 words in length from the introduction to the commencement of the bibliography.

A handwritten signature in black ink, appearing to read 'Hannah Marshall', with a stylized, cursive script.

Hannah Marshall

18<sup>th</sup> December 2015

## Abstract

### Chronic Probiotic Supplementation and its Effects on eHsp72 and LPS Concentration Following a Desert-based Ultramarathon

By

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This study investigated the effect of 12wk probiotic supplementation on the Lipopolysaccharides (LPS) and extracellular-heat-shock-protein 72 (eHsp72) response to a 7d ultra-endurance event (249.4km) in extreme heat [(average temperature  $\sim 38^{\circ}\text{C}$ ) Marathon des Sables (MDS) 2015]. Thirty-two (6 female) competitors were randomly allocated to receive probiotic, probiotic + glutamine, or no supplementation for 12wk prior to the MDS. Blood samples were collected on two occasions prior to the race [12wk (baseline) and 7d pre-race], and two further occasions post-race (6-8h and 7d post-race). Plasma eHsp72 and LPS concentrations were determined using ELISAs;  $\dot{V}\text{O}_{2\text{max}}$  was recorded at baseline and pre-race. A significant increase in overall mean  $\dot{V}\text{O}_{2\text{max}}$  was observed from baseline to pre-race ( $p < 0.05$ ), however no difference was found between groups ( $p > 0.05$ ). Overall mean post-race eHsp72 concentration was significantly increased ( $p < 0.05$ ) by 124% from baseline, there was no significant effect of group on eHsp72 concentration at any time point ( $p > 0.05$ ). There was no significant change in LPS concentration from baseline to post-race in all groups ( $p > 0.05$ ), no difference in LPS concentration was observed between groups at any time ( $p > 0.05$ ). This study indicates an ineffective role of PRO and PGLn supplementation on LPS translocation and eHsp72 response to ultramarathon performance in extreme heat.

**Key words:** Ultra-endurance; extracellular heat shock protein 72; lipopolysaccharides; probiotics

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## Published Work and Conference Presentations

### Published Work

- Taylor, L., Watkins, S., **Marshall, H.**, Dascombe, B. & Foster, J. (2015). The Impact of Different Environmental Conditions on Cognitive Function: A Focused Review. *Frontiers in Physiology*, 6.

### Conference Presentations

- **Marshall H.** & Taylor L. (2014). Does Raising Morning Core Temperature To Evening Levels Offset The Diurnal Variation In Measures Of Anaerobic Cycling Performance? BASES Student Conference – Portsmouth.



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## List of Abbreviations

°C	Degrees Celsius
ACSM	American College of Sports Medicine
ATP	Adenosine triphosphate
BASES	British Association of Sport and Exercise Sciences
b.min <sup>-1</sup>	Beats per minute
CON	Control group
d	Days
EDTA	Ethylenediaminetetraacetic acid
EHI	Exertional heat illness
EHS	Exertional heat stress
eHsp72	Extracellular heat shock protein 72
ELISA	Enzyme-linked immunosorbent assay
GI	Gastrointestinal
GLn	Glutamine
h	Hours
HPA	Hypothalamic-pituitary-adrenal
HR	Heart Rate
HSF-1	Heat shock factor-1
HSP	Heat shock protein
Hsp72	Heat shock protein 72
IgA	Immunoglobulin A
iHSP	Intracellular heat shock protein
Kcal	(Kilo) calories
kDa	Kilodaltons
Kg	Kilogram
LPS	Lipopolysaccharides
MDS	Marathon des Sables
Min	Minutes
ml.kg.min <sup>-1</sup>	Millilitres per kilogram per minute
O <sub>2</sub>	Oxygen
PGLn	Probiotic + Glutamine
PO	Power output
PRO	Probiotic
RH	Relative humidity
Rpm	Revolutions per minute
SAM	Sympatho-adrenalmedullary (SAM) axis,
SD	Standard deviation
T <sub>core</sub>	Core temperature
TT	Time Trial
TTE	Time to exhaustion
W	Watts
WBGT	Wet bulb globe temperature
Wk	Week
$\dot{V}O_2$	Volume of oxygen uptake
$\dot{V}O_{2max}$	Maximal volume of oxygen uptake
$\dot{V}O_{2Peak}$	Peak volume of oxygen uptake

## CHAPTER 1: General Introduction

Strenuous physical activity and/or extremes of environmental temperature can decrement physical (DeMartini *et al.*, 2014; Kark *et al.*, 1996; Peiffer and Abbiss, 2011) and cognitive performance (Taylor *et al.*, 2015), with their combination (exercise heat-stress) an acknowledged risk factor for development of exertional heat illnesses [EHI; (Armstrong *et al.*, 2007)]. Implicated within exertional heat stress (EHS) and EHI pathophysiologies are a plethora of physiological responses which initially act to protect the body from damage [e.g. heat shock protein (HSP) increases (Asea *et al.*, 2000) amongst others], yet if the exercise heat-stress is not resolved, negative physiological responses can be experienced, including the presentation of endotoxemia (Brock-Utne *et al.*, 1988; Ng *et al.*, 2008; Selkirk *et al.*, 2008). Athletes, military personnel, and occupational workers can be exposed to exercise heat-stress acutely or chronically dependent on the environment their prescribed task or pursuit is to be completed within.

The highly inducible isoform of the HSP 70 kDa family HSP72 [HSPA1A (Kampinga *et al.*, 2009)], at both the gene (Hsp) and protein (HSP) level, robustly increases in response to exercise heat-stress (Gibson *et al.*, 2015b; Périard *et al.*, 2012; Ruell *et al.*, 2014; Selkirk *et al.*, 2009), having a high affinity for denatured protein [e.g. heat-induced protein homeostasis disturbance (Mayer and Bukau, 2005)] and functions to resist thermal stress induced cell necrosis (Takayama *et al.*, 2003). The cytoprotective influence of Hsp72 [and other Hsps, e.g. Hsp27 (Concannon *et al.*, 2003) and HSP90 (Richter and Buchner, 2001)] principally involves the chaperoning and refolding of mis-folded and denatured proteins (Gabai and Sherman, 2002). Hsp72/HSP72 have been detected in both the intracellular (iHsp72) and extracellular (eHsp72) environments with ‘gene’ [typically mRNA (Gibson *et al.*, 2015a; Tuttle *et al.*, 2015)] Hsp72 released from the cell, whereas intracellular [iHSP (Périard *et al.*, 2015; Taylor *et al.*, 2010b)] and extracellular HSP [eHSP (Kampinga *et al.*, 2009; Tavaría *et al.*, 1996)] denote the protein within their respective environments. These iHSPs are well characterised as molecular chaperones, with iHsp72 and iHSP72 strongly correlating with *in vivo* cellular and whole body tolerance to thermal stress (Magalhaes *et al.*, 2010; McClung *et al.*,

2008; Selkirk *et al.*, 2009). In contrast to iHSP72, eHSP72 function *in vivo* is poorly characterised with high inter- and intra-individual variation, albeit increasing in response to a number of stressful situations, including exercise (Febbraio *et al.*, 2002a; Walsh *et al.*, 2001), heat-stress (Lovell *et al.*, 2007), exercise heat-stress (Gibson *et al.*, 2014) and trauma (Dybdahl *et al.*, 2002; Pittet *et al.*, 2002). The precise physiological role of eHSP72 is not yet fully understood, but is thought to play a crucial role in immunological function by initiating the secretion of pro-inflammatory cytokines (Tsan and Gao, 2004) and acting as a ‘danger signal’ to the immune system, thus offering increased cellular protection (Campisi *et al.*, 2003; Fleshner and Johnson, 2005).

The eHsp72 response to thermal (Lovell *et al.*, 2007; Gibson *et al.*, 2014) and exercise stress (Lancaster *et al.*, 2004; Walsh *et al.*, 2001) is documented, with their combination (exercise heat-stress) increasing the response further compared to exercise alone (Whitham *et al.*, 2007). Core temperature ( $T_{\text{core}}$ ) of  $>38.5^{\circ}\text{C}$  has demonstrated a strong relationship to the magnitude of eHsp72 response (Gibson *et al.*, 2014; Périard *et al.*, 2012; Ruell *et al.*, 2006). Endurance events also appear to initiate a substantial increase in eHsp72 expression due to their prolonged duration. Fehrenbach *et al.* (2005) demonstrated that a marathon run ( $260 \pm 39$  min at approximately  $65\% \dot{V}O_{2\text{max}}$ ) led to a  $> 2.5$ -fold increase in eHSP72 in comparison to a 120 min run at  $60\% \dot{V}O_{2\text{max}}$ , suggesting that exercise duration likely played a key role in the differential eHSP72 response seen. This was supported by Suzuki *et al.* (2006), whereby eHSP72 expression following an ironman triathlon (average finish time  $9 \text{ h } 59 \text{ min} \pm 0 \text{ h } 34 \text{ min}$ ) increased 22-fold from baseline [compared to  $> 2.5$ -fold increase post marathon (Fehrenbach *et al.*, 2005)], which was said to be as a function of exercise duration. The greater increase in eHSP72 following the ultramarathon (Suzuki *et al.*, 2006), in comparison with the marathon run (Fehrenbach *et al.*, 2005), supports the role of exercise duration as a key function of eHsp72/eHSP72 induction. Exercise duration (Suzuki *et al.*, 2006) therefore appears to be, alongside  $T_{\text{core}} >38.5^{\circ}\text{C}$  (Gibson *et al.*, 2014), a main function of increased eHsp72 release. This is interesting given the increasing popularity of physically demanding ultra-endurance events (Knechtle *et al.*, 2011); which are defined as races with distances  $>42$  km or durations  $>6$  hours [h; (Zaryski and Smith, 2005)]. Notable

events include the Jungle Marathon [>250 km over 6 days (d) within Brazilian rainforest], the Badwater Ultra (>200 km, Death Valley USA) and the Marathon des Sables [MDS (~250 km over 6 d within Sahara Desert)]. The extreme exercise heat-stress encountered by participants within such events can include temperatures >45°C (MDS) and relative humidity's (RH) >80% (Jungle Marathon); evidently placing competitors at an increased risk of developing EHI related pathophysiology.

Endurance exercise, especially in the heat, is often accompanied by gastrointestinal (GI) discomfort and is estimated at 30-90% prevalence within distance runners (Brouns and Beckers, 1993), with the possibility of mild to moderate symptomatology, including nausea, stomach cramps and bloating, and serious symptoms, such as haemorrhagic gastritis, hematochezia and ischemic bowel (Strid and Simrén, 2005). Exercise-induced GI discomfort and dysfunction is multi-factorial relative to aetiology, but is often primarily attributed to exercise heat-stress mediated hyperthermia *per se* and splanchnic hypoperfusion (Lambert, 2004; Moses, 2005), as blood is shunted away from the viscera and redirected to the heart, skin and skeletal muscles (Qamar and Read, 1987). Subsequent epithelial tight junction damage can occur, culminating in GI barrier dysfunction (Van Wijck *et al.*, 2011), allowing paracellular movement of lipopolysaccharides (LPS; endotoxins) into the bloodstream which can lead to endotoxemia (Bouchama and Knochel, 2002; Zuhl *et al.*, 2012). Yeh *et al.* (2013) noted a 54% increase in LPS concentration from baseline following 60 min running (70%  $\dot{V}O_{2max}$ , 33°C, 50% RH.), with Gill *et al.* (2015) demonstrating that 75% of participants in a 24 h continuous ultramarathon had at least one severe GI symptom which co-presented with a ~37% increase in circulating endotoxin concentration upon event completion compared to baseline. These increases (Gill *et al.*, 2015; Yeh *et al.*, 2013) are of concern as increased circulating levels of endotoxins can lead to fever, dizziness and GI distress, and may potentially result in sepsis and multi-organ damage (Van Leeuwen *et al.*, 1994). The aforementioned symptoms of endotoxemia are associated with decrements in overall performance (Brock-Utne *et al.*, 1988; Pfeiffer *et al.*, 2012), and it is therefore plausible to suggest that this will negate optimal recovery between demanding

endurance based exercise heat-stress bouts with limited recovery time (< 12 h), such as those experienced during the MDS.

Given that both endurance exercise (Fehrenbach *et al.*, 2005) and elevated  $T_{core} > 38.5^{\circ}\text{C}$  (Gibson *et al.*, 2014) are reportedly key functions for the increased eHsp72 concentration during exercise, it is extremely likely that undertaking an ultramarathon in extreme environmental conditions (e.g.  $>45^{\circ}\text{C}$ ) would initiate a significant eHsp72 response. In addition, increased circulating LPS, a common response to endurance performance (Brock-Utne *et al.*, 1988; Ng *et al.*, 2008), reportedly stimulates Hsp72 synthesis, in order to restore cellular homeostasis (Chan *et al.*, 2004; Hauser *et al.*, 1996; Lau *et al.*, 2000) and thus an amalgamation of the aforementioned stressors is expected to increase the expression of eHsp72 during ultramarathon performance. The subsequent and potentially protective physiological effect of increased eHsp72 expression on endotoxemia has been less commonly investigated. However, a modest increase in temperature (from  $37^{\circ}\text{C}$  to  $41^{\circ}\text{C}$ ) has been shown by Dokladny *et al.* (2006) to increase eHSP70 expression *in vitro*, leading to a subsequent protective effect on heat-induced tight junction barrier disturbance; suggesting elevated eHSP72 may reduce GI barrier permeability and thus theoretically could reduce endotoxemia [this is important given that endotoxemia has been shown to decrement physical performance (Pfeiffer *et al.*, 2012)]. However, research in this area is sparse, and further investigation is required to fully understand the effect of increased circulating LPS (and the possible protective influence of increased eHSP70/72 concentrations) in human exercise models *per se*, particularly for exercise models like the MDS.

Evidently, methods to reduce GI permeability and the subsequent risk of endotoxemia during long duration exercise are required to ensure optimal performance and recovery (including between bouts) is achieved. Nutritional interventions have been investigated, such as bovine colostrum ingestion (Marchbank *et al.*, 2011; Morrison *et al.*, 2014), glutamine supplementation (Lambert *et al.*, 2001; Zuhl *et al.*, 2015), fluid replacement (Lambert *et al.*, 2001), and ascorbic acid supplementation (Ashton *et al.*, 2003), but have shown equivocal results. Probiotics [defined as ‘living microorganisms which upon ingestion in certain numbers exert health benefits beyond general nutrition’ (Guarner and

Schaafsma, 1998)] have recently gained interest due to the potential benefits on the gut and gut barrier function. Specifically, probiotic supplementation has been shown to enhance defence against infection via increased strength of tight junction proteins, maintenance of cellular polarization and mucosal homeostasis (Mengheri, 2008; Otczyk and Cripps, 2010; Sherman *et al.*, 2009). Kekkonen *et al.* (2007) demonstrated a reduction in GI-symptoms in marathon runners following chronic probiotic supplementation, however the data regarding the effect in ultra-endurance events is not currently available. Given such events can last >24 h and have limited recovery time between demanding exercise heat-stress bouts (<12 h), competitors are likely to experience elevated eHsp72 expression (Fehrenbach *et al.*, 2005; Périard *et al.*, 2012), and are at increased risk of GI discomfort during performance (Gill *et al.*, 2015). As a result, methods to attenuate detrimental GI-related side effects are required to optimize performance in ultra-endurance based exercise (i.e. during the MDS). This research therefore aims to investigate the effect of 12 weeks (wk) of chronic probiotic supplementation prior to an ultra-endurance event, and its effects on LPS translocation, GI discomfort and any subsequent eHsp72 response.

## CHAPTER 2: Literature Review

### 2.1 Heat stress and thermoregulation

Humans are homeothermic and attempt to maintain an optimal  $T_{core}$  of  $\sim 37^{\circ}\text{C}$  through mechanisms of heat loss and heat gain (Benzinger, 1969; González-Alonso, 2012). Heat exchange occurs at all times in humans to ensure that an optimal  $T_{core}$  is maintained. The heat balance equation (Figure 2.1), derived from the first law of thermodynamics, models the rate of heat storage utilizing the four major mechanisms of heat exchange: conduction ( $\dot{C}$ ), convection ( $\dot{K}$ ), radiation ( $\dot{R}$ ) and evaporation ( $\dot{E}$ ), and is as follows (Cheung, 2009):

$$\dot{S} = \dot{M} \pm \dot{W}_k \pm \dot{R} \pm \dot{C} \pm \dot{K} - \dot{E} \text{ (W.m}^{-2}\text{)}$$

**Figure 2.1.** The heat balance equation (Cheung *et al.*, 2000).



$\dot{M}$  refers to metabolic heat production, and  $\dot{W}_k$  refers to the external work performed by the individual. A positive value for  $\dot{S}$  represents elevated body heat storage, which could eventually lead to hyperthermia (Cheung *et al.*, 2000).

Thermal homeostasis is required in order to ensure that optimal molecular, cellular, physical and cognitive function is maintained (Nakamura and Morrison, 2008; Bandelow *et al.*, 2010; Parker *et al.*, 2013; Sawka and Montain, 2000). During exercise, especially in extreme environments (e.g. MDS),  $T_{\text{core}}$  increases (González-Alonso *et al.*, 1999; Nielsen *et al.*, 1997) and thus physiological methods to promote heat loss are required (Cheung *et al.*, 2000). Thermosensitive neurons in the preoptic-anterior hypothalamus detect increases in  $T_{\text{core}}$ , and receive afferent sensory input from thermoreceptors throughout the body [including the spinal cord, internal organs and the skin (Wendt *et al.*, 2007)]. This allows the hypothalamus to initiate the most appropriate thermoregulatory response to the thermal stress, in an attempt to regulate  $T_{\text{core}}$  at the optimal  $\sim 37^\circ\text{C}$  (Benzinger, 1969).

When exposed to extreme conditions, such as hot or cold, the human body will exhibit physiological changes in order to regulate temperature at the optimal level (Cheung, 2009). One key physiological change that occurs is redistribution of blood flow to reduce skin temperature, or sweating to increase evaporative heat loss (Sawka *et al.*, 2011). Elevated  $T_{\text{core}}$  leads to heat transfer from the core to the skin, to allow the dissipation of heat to the environment via vasodilation and the aforementioned mechanisms of heat loss (Sawka *et al.*, 2011). Approximately 50-80% of the heat flow in the tissue is carried in or out by the blood flow, and it is said that human  $T_{\text{core}}$  would rise by  $12^\circ\text{C}$  in an hour if heat loss via blood flow did not occur (Acharya *et al.*, 2014). Skin blood flow can increase by  $\sim 6\text{-}8\text{ L/min}$  via thermoregulatory vasodilation as a result of hyperthermia (Charkoudian, 2003). This elevation requires the redistribution of blood to the skin from other areas exhibiting a lower demand for blood flow during performance, such as the splanchnic region (Clausen, 1977).

## 2.2 Exercise in the heat

Exercise in the heat exerts severe levels of physiological stress upon the body, due to the increased cardiovascular and thermoregulatory demands (González-Alonso *et al.*, 2008). Maintenance of thermoregulatory homeostasis is more complex during exercise heat-stress; due to the increase in metabolic heat production (principally from the active skeletal muscle) combined with the impairment of heat loss mechanisms, resulting in elevated  $T_{\text{core}}$  [see Figure 2.1 (Gleeson, 1998; Nybo, 2008)]. This rise in  $T_{\text{core}}$ , coupled with the aforementioned (section 2.1) competition for limited blood flow (Tan and Lee, 2015), may ultimately impair endurance performance (Nybo *et al.*, 2014), and can lead to exercise heat-stress induced illness, such as hyperthermia, GI discomfort and endotoxemia (Brock-Utne *et al.*, 1988).

A plethora of research is available to demonstrate the detrimental effects of heat exposure on exercise performance across a variety of exercise types (Parkin *et al.*, 1999; Wingo *et al.*, 2005), particularly prolonged duration endurance exercise, due to extended exposure to extreme heat (Ely *et al.*, 2007; Peiffer and Abbiss, 2011). An increased  $T_{\text{core}}$  combined with elevated exogenous heat stress can lead to deteriorations in performance (Nybo *et al.*, 2014), and several studies (Galloway and Maughan, 1997; Lorenzo *et al.*, 2010; Tatterson *et al.*, 2000; Tucker *et al.*, 2004) have demonstrated the impact of exogenous heat on performance, indicating a reduction in exercise capacity in hot conditions. Endurance running imposes great levels of physiological and mechanical stress upon the body due to the ‘whole body’ nature of the exercise (Dawson *et al.*, 1985), and as a result, performance capacity is significantly impaired. It is possible that this decline in performance is related to the increased risk of heat related illness during exercise-heat stress. Such illness, including hyperthermia (Nybo *et al.*, 2014) and gut related discomfort and dysfunction (Lambert, 2004) may induce further illness, such as endotoxemia (Brock-Utne *et al.*, 1988), and can result in both central and peripheral fatigue (Cheung and Sleivert, 2004; Supinski *et al.*, 2000), and thus subsequently impair performance and recovery [including between consecutive (i.e. < 12 h) exercise bouts]. Additionally, athletes undertaking ultra-endurance exercise are at an increased risk of heat-related illness due to the prolonged exposure to unfavourable conditions, which can exceed 24 h (Gill *et al.*, 2015).

### 2.2.1 The Marathon des Sables

Ultra-endurance events are becoming increasingly popular (Knechtle *et al.*, 2011). The MDS, a footrace covering > 250 km across the Sahara Desert over 7 d, is an ultramarathon often referred to as ‘the toughest footrace on earth’. Competitors are required to carry all of their own equipment and food, must consume at least 1,500 kcal per day, and are rationed to ~ 9 L of water per day. Undertaking ultra-endurance events in extreme environments can place individuals at extreme risk of heat-related illness, such as hyperthermia, and may also increase the risk of gut related discomfort, which could consequently impair performance (Edler *et al.*, 2014; Sawka, 2004). These side effects could prove severely detrimental in events, such as the MDS, where within race recovery between stages is vital. The mechanistic physiological responses to target in an attempt to reduce the risk of such illness (including increased HSP72 concentration, amongst others) will be discussed in further detail in section 2.3.

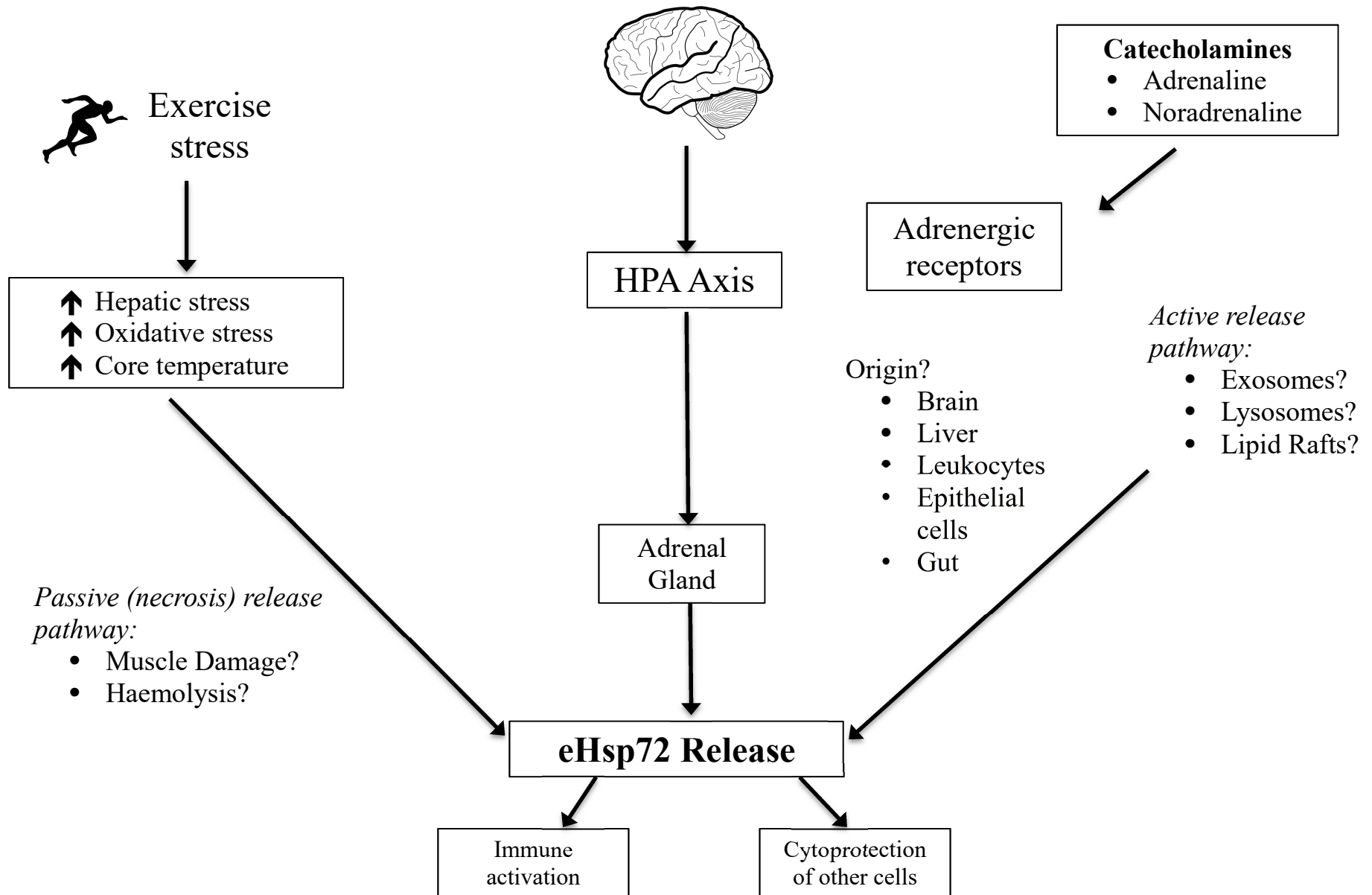
## 2.3 Specific responses to exercise heat-stress

### 2.3.1 The molecular response: Extracellular heat shock proteins

As detailed in section 2.2, exercise heat-stress places the human body under severe levels of stress (González-Alonso *et al.*, 2008; Nybo, 2008). Protective physiological responses, acute and chronic in nature, are required to reduce the risk posed by exercise and exercise heat-stress, and to prevent potential cellular and tissue damage (Morimoto and Santoro, 1998). Heat shock proteins (also frequently termed stress proteins) are a family of highly conserved proteins produced in response to conditions of extreme stress (Heck *et al.*, 2012; Whitley *et al.*, 1999). It is highly accepted that iHsp72 is found in the majority of the body’s cells, and is upregulated in response to cellular and organismic stressors (Hartl, 1996). However, research into the role of eHsp72 has only recently gained interest. In a clinical setting, Pockley *et al.* (2002) demonstrated that individuals suffering with disease states such as atherosclerosis (Pockley *et al.*, 2003), hypertension (Pockley *et al.*, 2002), and renal disease (Wright *et al.*, 2000) had chronically higher basal eHsp72 levels in comparison to healthy age-matched controls. Following these findings, Walsh *et al.*

(2001) and Febbraio *et al.* (2002a) reported a rapid increase in the concentration of eHsp72 after exposure to acute stressors in human cells in the absence of clinical disease states. As a result, these studies were the first to recognise eHsp72 release as a feature of the normal stress response, with the increased eHsp72 concentration suggested to activate the immune response (Moseley, 2000). Acute stressors, including psychological stress (Fleshner *et al.*, 2004), trauma (Pittet *et al.*, 2002), exercise-stress (Lancaster *et al.*, 2004) and heat stress (Lovell *et al.*, 2007) induce an increase in eHsp72 concentration in both human and animal models.

The exact mechanism of exercise-induced eHsp72 release is currently not fully understood, however two mechanisms have been suggested, dependent on the mode, intensity and/or duration of exercise: i) through cellular necrosis [muscle damage (Suzuki *et al.*, 2006) or haemolysis (Whitham and Fortes, 2006)]; or ii) through an active exocytosis mechanism (Lancaster and Febbraio, 2005; Mambula *et al.*, 2007). Cell lysis or death can lead to the passive release of HSP72 into the extracellular environment (Basu *et al.*, 2000; Fleshner and Johnson, 2005). HSP72 is released into the extracellular milieu following cell/tissue necrosis, causing the protein content of the cell to ‘spill’ into the surrounding space (Whitham and Fortes, 2008). Exercise can cause damage to the sarcolemma, which reflects necrosis, and may contribute to the exercise-induced increase in eHSP72 (Fehrenbach *et al.*, 2005). However a direct correlation between eHsp72 and markers of muscle damage is yet to be reported, thus these mechanisms remain speculative (Whitham and Fortes, 2008). The release of hormones from the pituitary gland via the hypothalamic-pituitary-adrenal (HPA) axis, or sympatho-adrenalmedullary (SAM) axis, is activated by a stress response caused following the exposure to stressful exercise-induced stimuli, such as increased oxidative stress (Fischer *et al.*, 2006) and  $T_{core}$  (Ruell *et al.*, 2006). The origin of eHsp72 is currently unclear, but is thought to involve lipid rafts, exosomes and lysosomes (Lancaster and Febbraio, 2005). Figure 2.2, from Whitham and Fortes (2008), demonstrates a proposed model for *in vivo* human exercise-induced release of eHsp72.



**Figure 2.2.** Model for the exercise-induced release of eHsp72 [Adapted from Whitham and Fortes (2008)].

### 2.3.1.2 Exercise and the heat shock response

As stated in section 2.3.1, exercise has been shown to trigger an increase in eHSP72 concentration. Exercise causes a disturbance to cellular homeostasis and places the body under increased stress, instigating a subsequent need for increased eHSP72 concentration for cellular protection (Kregel, 2002). Moseley (1997) stated that the heat strain imposed upon the body during heat stress is reduced via enhanced HSP72 synthesis. A plethora of evidence is available to support this theory, and demonstrates the increase in eHSP72/eHsp72 caused by exercise stress (see Table 2.1).

Endurance exercise has been shown to induce a significant increase in eHsp72 concentration. In temperate conditions, Walsh *et al.* (2001) were the first to demonstrate an exercise-induced release of eHsp72, whereby an increase from  $0.13 \pm 0.10$  ng/mL at rest to  $1.02 \pm 0.41$  ng/mL immediately post-exercise was found following 60 min treadmill running at 70%  $\dot{V}O_{2peak}$ . Lancaster *et al.* (2004) supported these findings, showing an average eHsp72 increase of  $\sim 1.00 \pm 0.38$  ng/mL from pre- to post-exercise following 180 min cycling at 60%  $\dot{V}O_{2max}$ . Additional research (as detailed in Table 2.1) has reinforced this evidence across a variety of exercise modalities, durations and intensities.

Fehrenbach *et al.* (2005) demonstrated a duration- and intensity- dependent role for exercise-induced eHSP72 release, whereby prolonged duration exercise, a marathon run ( $260 \pm 39$  min) at 60%  $\dot{V}O_{2max}$ , led to a greater eHSP72 concentration immediately post-exercise ( $16.3 \pm 12.1$  ng/ml) in comparison with a shorter duration run (120 min) at the same intensity ( $4.2 \pm 2.2$  ng/ml). The significantly higher eHSP72 concentration following the marathon run suggests a key role for exercise duration in eHSP72 production. Furthermore, exercise in extreme environmental conditions has been shown to initiate a larger eHsp72 response (Table 2.1). Gibson *et al.* (2014) clearly demonstrated this effect through 90 min cycling at 50%  $\dot{V}O_{2peak}$  in three experimental conditions. Following exercise in extreme heat (40°C, 37% RH), eHsp72 concentration was increased by 172.4% from baseline, whereas the same exercise at 30.2°C, 51% RH led to an eHsp72 increase of just 25.7%. It was

suggested that these changes were modulated by large, rapid changes in  $T_{\text{core}}$ , with the greatest increase displayed during exercise at 40°C, therefore demonstrating a significant role of exogenous and endogenous heat stress on eHsp72 concentration. It was suggested that  $T_{\text{core}}$  of >38.5°C was a key threshold for the increase in eHsp72 concentration (Amorim *et al.*, 2008). Périard *et al.* (2012) reinforced this initial data, and supported the notion of a  $T_{\text{core}}$  threshold for eHsp72 release (Gibson *et al.*, 2014), whereby  $T_{\text{core}}$  >38.5°C, when combined with exercise stress, was shown as the most potent stimuli for increased eHsp72 concentration, and indicated the importance of exercise intensity and duration as modulators of eHsp72 release. A significant correlation was found between eHsp72 and attained  $T_{\text{core}}$  following cycling to exhaustion at 60%  $\dot{V}O_{2\text{max}}$  in 40°C, 50% RH; and rate of increase in  $T_{\text{core}}$  at 75%  $\dot{V}O_{2\text{max}}$  in the same conditions.

#### 2.3.1.3 Ultra-endurance and the heat shock response

Evidently, exercise duration is a key modulator of eHsp72 release (Amorim *et al.*, 2008; Febbraio *et al.*, 2002b; Marshall *et al.*, 2006), and thus ultra-endurance events, defined as lasting >6 h (Zaryski and Smith, 2005), are likely to induce a significant increase in eHsp72 concentration. Of the limited research available regarding ultra-endurance events, evidence thus far has demonstrated a significant post-exercise increase in eHSP70 concentration. An ironman triathlon, consisting of a 3.8 km swim and a 180 km cycle followed by a 42.2 km run in moderate ambient temperatures (23.3°C ± 1.9°C), with an average finish time of 9 h 59 min ± 0 h 34 min, led to a 22-fold increase in plasma eHSP70 from pre- to post-race (Suzuki *et al.*, 2006). Gomez-Merino *et al.* (2006) compared the eHSP70 response to two ultra-endurance events: a long distance triathlon (4 km swim, 120 km cycle, 30 km run) in 12 trained male triathletes, and a 100 km run in 12 trained male ultramarathoners, in similar ambient temperatures, (24.1°C and 25.5°C in the triathlon and run, respectively). Interestingly, eHSP70 concentration following the 100 km run (16.74 ± 0.64 ng/mL) was 173% higher than eHSP70 concentration following the long distance triathlon (6.13 ± 0.61 ng/mL), despite no significant difference in exercise duration between events. Gomez-Merino *et al.* (2006) postulated that the greater disruption of cellular homeostasis caused by the 100 km run elicited a greater

eHSP70 response, which was thought to be upregulated as a method of cellular protection. This is an interesting finding suggesting that ultra-endurance running (as opposed to swimming or cycling) may lead to the greatest change in eHSP70 concentration, due to the increased degree of whole body stress (Dawson *et al.*, 1985; Rehrer *et al.*, 1989), potentially leading to increased membrane disturbance/damage (Oktedalen *et al.*, 1992) and thus an increase in eHsp72 concentration through the aforementioned (section 2.4.1) necrosis-related release mechanism (Suzuki *et al.*, 2006). The two events completed in this study were undertaken in warm environments [peak: 23°C (Suzuki *et al.*, 2006) and 24.1°C (Gomez-Merino *et al.*, 2006)], thus it is highly likely that undertaking ultra-endurance running in hot conditions (>30°C), such as those experienced in the MDS, will initiate an even greater eHSP70 response, due to the combined effects of exercise and heat stress.



**Table 2.1.** An overview of studies investigating the effect of exercise and exercise heat-stress on eHsp72 concentration.

Author (year)	Subjects	Study design	Temperature	Blood sampling	Effect on eHSP72 (mean $\pm$ SD)
<b><u>Exercise stress only</u></b>					
Walsh <i>et al.</i> (2001)	6 subjects, male n=5, female n=1	60 minutes treadmill running (70% $\dot{V}O_{2peak}$ )	20°C, 40% RH	Intravenous blood sample from forearm. [Pre-, 30 min (during) and Immediately-, 2h- and 8h- post-exercise]	$\uparrow$ Serum Hsp72: Rest: $0.13 \pm 0.10$ ng/mL 30 min (during): $0.87 \pm 0.24$ ng/mL Immediately post: $1.02 \pm 0.41$ ng/mL
Febbraio <i>et al.</i> (2002b)	7 healthy, active males (19-33 years)	2.5h 2-legged leg extensor exercise (40% $W_{max}$ ). Glycogen depletion trial 24h pre-exercise. Main trial: 4-5h (until exhaustion) at 40% $W_{max}$ . Comparing HSP in depleted leg (DL) vs control leg (CL)	Not stated	Femoral artery and vein in right leg, femoral vein in left leg cannulated. Blood sample taken at rest, 1h hour intervals during exercise.	DL: $\uparrow$ HSP72 ~2 –fold immediately after exercise CL: $\leftrightarrow$ HSP72
Lancaster <i>et al.</i> (2004)	6 healthy endurance trained males	180 minutes cycling (60% $\dot{V}O_{2max}$ ). Consumed ~250ml CHO solution every 15 min.	21°C $\pm$ 1°C	Catheter in internal jugular and radial artery, samples taken pre- and immediately post-exercise for measures of cerebral Hsp72.	$\uparrow$ Arterial serum Hsp72 in 4 of the 6 subjects by $\sim 1.00 \pm 0.38$ ng/ml

Ruell <i>et al.</i> (2006)	Male runners with (EHI, n = 22) and without (CON, n=7) previous history of exertional heat illness.	14km “Surf to City” run, Sydney.	21°C, 33% RH	Venous blood samples within 10 min of completion (CON) or immediately upon admission to medical facility (EHI).	↑ Plasma eHsp72 in severe EHI vs mild EHI immediately post-exercise.
Whitham <i>et al.</i> (2006)	10 healthy, male, endurance trained cyclists	90 min cycling ( $74 \pm 1\% \dot{V}O_{2max}$ ) following consumption of 6 ml/kg body mass 1) Caffeine or 2) Placebo.	$21.1 \pm 0.8^{\circ}\text{C}$ , $52 \pm 5\%$ RH	Venous blood samples for plasma HSP72: Pre-supplement, pre-, immediately post-, 1h post-exercise.	↑ HSP72 in both groups compared to baseline. ↑ HSP72 greater in caffeine compared with placebo group.
<b><u>Exercise heat-stress</u></b>					
Marshall <i>et al.</i> (2006)	7 non-heat acclimated males	2 consecutive days cycling at $42.5\% \dot{V}O_{2max}$	38°C, 60% RH	Venous blood sample from antecubital vein, pre-, immediately post- and 22h post- exercise using cannulation	During exercise: ↑ eHSP72 ~1.26ng/ml. 2 hours of passive heating (38°C): ↔ eHSP72.
Whitham <i>et al.</i> (2007)	11 moderately trained males	2h immersion/ deep water running ( $58\% \dot{V}O_{2max}$ ). Exercise induced heat (EIH), Clamped exercise	Water temp: EIH: $35.3 \pm 0.9^{\circ}\text{C}$ CLex: $23.5 \pm 0.9^{\circ}\text{C}$	Venous blood samples for plasma eHSP72: pre-, immediately post- and 60min post- exercise.	All trials: significantly ↑ eHSP72. Exercise heat-stress = greatest ↑ eHSP72.

		(CLex) Passive heat (PH) control (CON).	PH: $38.5 \pm 0.2^{\circ}\text{C}$ CON: $35.3 \pm 0.2^{\circ}\text{C}$		
Amorim <i>et al.</i> (2008)	9 heat-acclimated subjects. Male, n=7 Female n=2	Repeated measures, counterbalanced design. Heat acclimation- 10d walking/running at 56% $\dot{V}O_{2\text{peak}}$ for 100min ( $42^{\circ}\text{C}$ , 30% RH). 3d post-acclimation: 2x heat stress trials at a low (LS) or high (HS) rate of heat storage.	$42^{\circ}\text{C}$ , 30% RH	Venous blood sample from antecubital vein pre- and post-exercise.	$\uparrow$ eHsp72 from pre- to post-exercise in both conditions.
Périard <i>et al.</i> (2012)	16 males	Cycle to exhaustion (60% vs 75% $\dot{V}O_{2\text{max}}$ )	$40^{\circ}\text{C}$ , 50% RH	Venous blood samples: rest, 10min, 30min, exhaustion, and 24h post.	$\uparrow$ eHsp72 in both conditions following exercise to exhaustion
Gibson <i>et al.</i> (2014)	10 healthy males	90min cycling (50% $\dot{V}O_{2\text{peak}}$ ) Repeated measures: 1) $20^{\circ}\text{C}$ , 63% RH (TEMP) 2) $30.2^{\circ}\text{C}$ , 51% RH (HOT) 3) $40^{\circ}\text{C}$ , 37% RH (VHOT)	1) $20^{\circ}\text{C}$ , 63% RH (TEMP) 2) $30.2^{\circ}\text{C}$ , 51% RH (HOT) 3) $40^{\circ}\text{C}$ , 37% RH (VHOT)	Venous blood samples for plasma eHsp72 taken pre-, immediately post- and 24h post- exercise.	VHOT: $\uparrow$ eHsp72 +172.4% HOT: $\uparrow$ eHsp72 + 25.7% ( $p > 0.05$ ) TEMP: $\downarrow$ eHsp72 - 1.9% ( $p > 0.05$ )

Ruell <i>et al.</i> (2014)	14 male runners with (n=7) and without (n=7) previous history of exertional heat illness	60 min treadmill run (72% $\dot{V}O_{2max}$ ).	30°C, 40% RH	Cannula inserted into antecubital vein and blood samples taken every 10 min for plasma eHSP72	↑ Plasma eHSP72: CON: 1.18±0.14 ng/ml (rest), 4.56 ± 0.63 ng/ml (60 min) EHI: 0.86 ± 0.08 ng/ml (rest), 4.04 ± 0.45 ng/ml (60 min).
Taylor <i>et al.</i> (in press)	6 males	Randomised double-blind crossover design. High intensity running protocol” 20 x 10s runs (23.0 ± 1.8 km/h <sup>-1</sup> ). 7 d effective microorganism –x supplementation or 7 d placebo followed by 1 HOT and 1 TEMP.	TEMP: 20.4°C HOT: 34.7°C	Venous blood sample taken from antecubital fossa	↑ Plasma eHsp72 pre- to post-exercise. ↑ eHsp72 was moderated by temperature. TEMP: ↑ eHsp72 + 0.8 ng/ml, HOT: ↑ eHsp72 +1.5ng/ml (↑ 418%).
<b><u>Ultra-endurance exercise</u></b>					
Gomez-Merino <i>et al.</i> (2006)	12 male triathletes vs 12 male ultramarathoners	Triathlon (4 km swim, 120 km cycle, 30 km run) vs 100 km run	Triathlon: peak 24.1°C Ultramarathon: peak 25.5°C	Venous blood sample	↑ eHSP72 following triathlon (6.13 ± 61ng/ml <sup>-1</sup> ) and ultramarathon (16.74 ± 4.34 ng/ml <sup>-1</sup> ). 173% greater ↑ in runners vs triathletes.

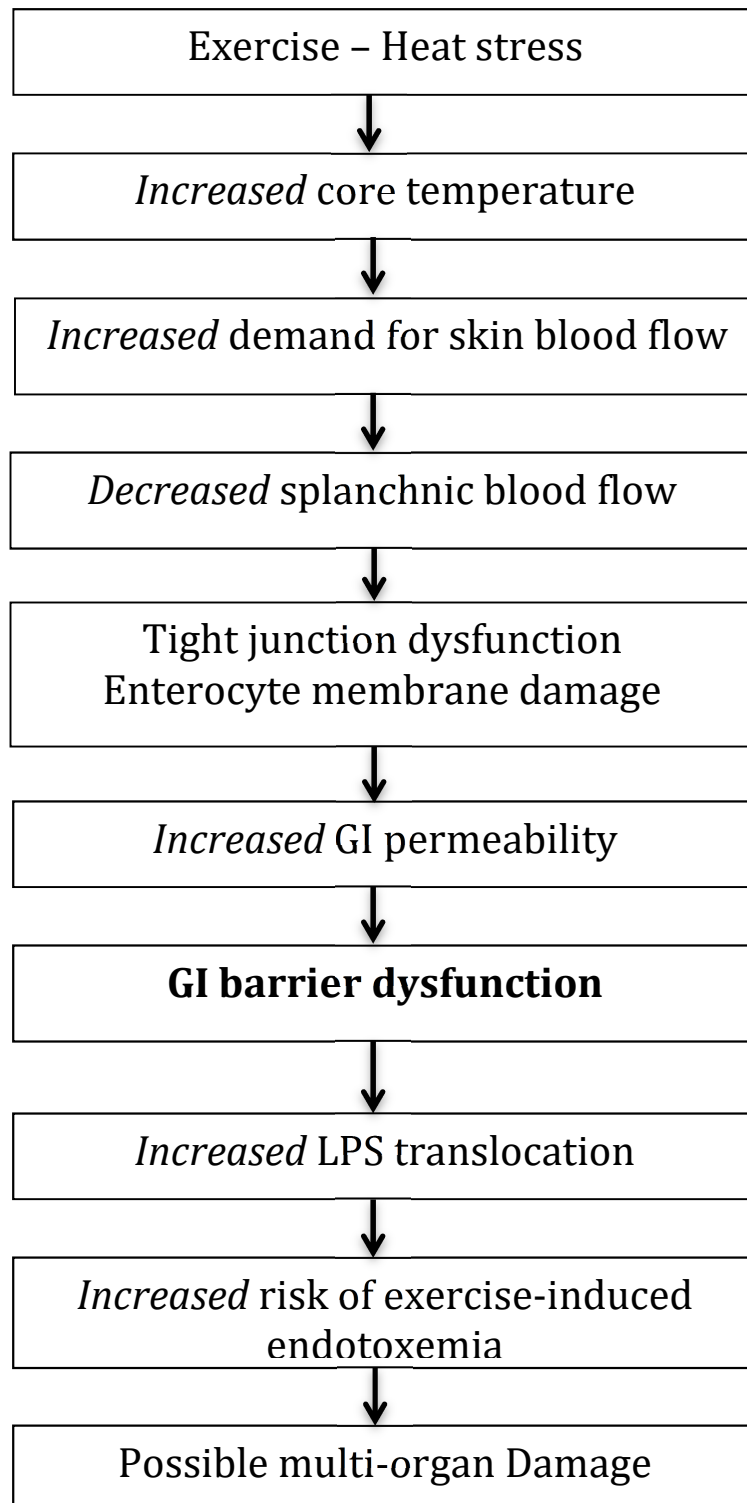
Suzuki <i>et al.</i> (2006)	9 male triathletes	Ironman Triathlon (3.8km swim, 180km cycle, 42.2km run)	23.3 ± 1.9°C 60 ± 14% RH	Venous blood sample, 2 days pre-race, within 30min post-race	↑ Plasma HSP70 2200% from baseline to post-race
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↑ = increase; ↓ = decrease; ↔ = no change.

### 2.3.2 The gastrointestinal response

At rest 20% of the cardiac output is received by the splanchnic organs, but only 10-20% of the available oxygen ( $O_2$ ) is consumed (Rowell *et al.*, 1964). During exercise,  $O_2$  demand is significantly increased, resulting in the redistribution of blood to the organs, skin and working muscles and away from the intestines (Qamar and Read, 1987). Research has demonstrated significant reductions in gut blood flow during exercise. Cycling at 70%  $\dot{V}O_{2max}$  for 60 min led to a reduction in splanchnic blood flow of between 50% (Peters *et al.*, 2001) and 80% (Rehrer *et al.*, 2001), with reductions as big as 87% seen following exercise at 97%  $\dot{V}O_{2max}$  (Rowell *et al.*, 1964). Reduced splanchnic blood flow can lead to gut ischemia, with a reduction in small intestinal blood supply of > 50% shown to induce detectable mucosal tissue injury (Bulkley *et al.*, 1985), which can consequently lead to GI related discomfort and dysfunction.

Damage to the epithelial tight junctions and intestinal walls as a result of gut ischemia (and other mechanisms) can increase intestinal permeability and result in GI barrier dysfunction (Van Wijck *et al.*, 2011). This increase in permeability can allow undesirable translocation of LPS from the gut into the systemic circulation (Berkes *et al.*, 2003), which can lead to symptoms including nausea, fever, dizziness and possible endotoxemia, should LPS concentration increase to > 5 pg/ml (Schippers *et al.*, 2008). Figure 2.3 [adapted from Lambert (2004)] displays the flow of events leading to GI barrier dysfunction and endotoxemia as a result of exercise heat-stress.



**Figure 2.3.** The flow of events leading to GI barrier dysfunction, and the possible consequences of such disruption [Adapted from Lambert (2004)].

### 2.3.2.1 Exercise and the gastrointestinal system

During exercise, GI discomfort can lead to impaired endurance performance (Rehrer *et al.*, 1989). Riddoch and Trinick (1988) demonstrated that 83% of 471 marathon runners suffered from GI disturbances during or immediately after running, and 29% of runners felt that their performance had been adversely affected by these symptoms. During exercise in the heat,  $T_{\text{core}}$  increases at a faster rate, which leads to an increased demand for skin blood flow for heat dissipation and therefore a subsequent reduction in splanchnic blood flow, consequently placing the individual at a greater risk of exercise-induced endotoxemia and GI discomfort (Otte *et al.*, 2001; van Wijck *et al.*, 2012).

Following 60 min running at 70%  $\dot{V}O_{2\text{max}}$  in 33°C, 50% RH, LPS concentration increased by 54%, whereas no significant increase in LPS concentration was shown following the same exercise in 25°C, 60% RH (Yeh *et al.*, 2013), indicating a role of heat stress upon LPS translocation. Furthermore, Ng *et al.* (2008) reported a 31.6% increase in LPS concentration following a 21 km road race in warm and humid conditions (exact temperatures not reported). Whilst only 4 of the 30 runners studied by Ng *et al.* (2008) met the criteria for mild endotoxemia (LPS concentration > 5 pg/ml), it is important to recognise the significant increase in LPS concentration caused by exercise heat-stress. GI discomfort data was not reported in these studies (Ng *et al.*, 2008; Yeh *et al.*, 2013), however elevated LPS concentration has previously been shown to relate to GI discomfort (Bosenberg *et al.*, 1988; Brock-Utne *et al.*, 1988), and thus future research should investigate this relationship further, particularly under conditions of extreme heat stress.

Moreover, ultra-endurance events also reportedly lead to significant levels of GI discomfort; Stuenkel *et al.* (2013) reported nausea, abdominal cramps, diarrhoea and vomiting from 9 of 15 runners following a 161 km ultramarathon. Whilst LPS was not recorded and thus details of GI damage cannot be elucidated, it is clear that GI discomfort was a common occurrence. Jeukendrup *et al.* (2000) also demonstrated the GI response to ultra-endurance exercise following an ironman triathlon (3.8 km swim, 185 km cycle, 42.2 km run) in conditions peaking at 32.1°C.



Of 29 athletes, 93% reported symptoms of GI discomfort, with 7% forced to abandon the race due to severe GI distress (vomiting and diarrhoea). In addition, elevated LPS concentration was detected in 68% of athletes, and although only mild endotoxemia (5 – 15 pg/ml) was observed, this demonstrated the effect of ultra-endurance exercise on the GI system. The impact of ultra-endurance exercise has previously been established by Brock-Utne *et al.* (1988), whereby 80% of 89 runners experienced endotoxemia (LPS > 100 pg/ml) following an 89.4 km race, and 2% experienced lethal concentrations of LPS (> 1000 pg/ml). It is suggested that exercise modality may influence the degree of discomfort; as the increased stress caused by the up and down motion of running may induce a greater degree of GI damage (Brouns and Beckers, 1993). Brock-Utne *et al.* (1988) also demonstrated that 80.6% of runners with elevated plasma LPS concentration reported GI symptoms including nausea, vomiting and/or diarrhoea, whereas only 17.7% of runners with low LPS concentration reported these symptoms. Although less research has been conducted regarding the GI response to ultra-endurance exercise, existing knowledge indicates the possibility of a key relationship between LPS concentration and GI discomfort during ultra-endurance running performance, particularly in hot environments.

As stated in section 2.2.3, the MDS is classed as one of the toughest ultramarathons in the world, consisting of 7 d consecutive exercise, with the longest stage requiring competitors to cover ~ 80 km in 24 h, in the heat of the Sahara Desert (reaching up to 50°C). This substantial level of demand placed upon the human body will likely cause a significant increase in  $T_{core}$ , leading to potential gut ischemia and subsequent LPS translocation and GI distress. However, research into the effect of the MDS upon GI discomfort has not yet been conducted, thus will be of great interest to practitioners to comprehend the damage that can occur during an ultramarathon in such extreme environmental conditions, and consider plausible interventions to offset this potentially serious medical condition.

### 2.3.3 LPS and eHsp72

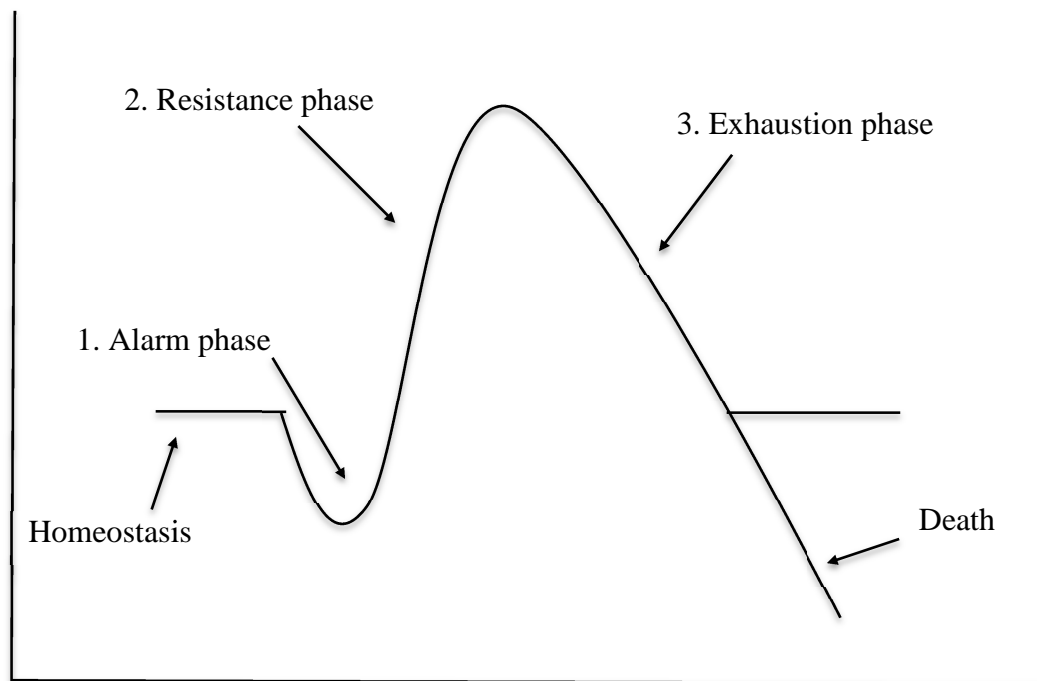
The relationship between circulating LPS and eHsp72 is still incompletely understood. Animal studies have demonstrated a clear, protective effect of eHsp72 in

septic animals *in vivo* (Lin *et al.*, 2010; Wischmeyer *et al.*, 2001), through a reduction in pro-inflammatory cytokine release (Singleton and Wischmeyer, 2007) and enhanced cell survival (Zhao *et al.*, 2012). A review of 41 animal studies by Briassoulis *et al.* (2014) demonstrated a 95.8% level of protection by both intra- and extra-cellular Hsp72 against sepsis. The induction of eHsp72 demonstrated a strong protective effect, and led to enhanced survival in all septic animal models.

However, the role of eHsp72 in response to elevated circulatory LPS concentration in humans is currently somewhat inconclusive. Selkirk *et al.* (2009) demonstrated a protective role of eHsp72, whereas McConnell *et al.* (2011) indicated a possible relation of eHsp72 to mortality and infection. Of 14 human studies analysed by Briassoulis *et al.* (2014), only 50% showed a protective eHsp72 response, and 14.3% demonstrated a non-protective eHsp72 response to sepsis. Tulapurkar *et al.* (2015) demonstrated that *in vitro* treatment with LPS for 6 h in human THP-1 cells stimulated eHsp70 release, which indicates a possible protective role of eHsp72 against the stress induced by elevated LPS concentration. However, further research into this relationship is required to develop a greater understanding regarding the role of eHsp72 against this increased level of stress.

It is plausible that an ‘inverted U’ stress response, in line with the classic Hans Selye (1946) General Adaptation Syndrome (GAS), could be seen in response to changes in LPS concentration. This theory detailed a three-phase response to stress in humans (Figure 2.4), whereby a protective effect is demonstrated in response to an appropriate amount of stress, however too much stress can be severely detrimental: i) phase 1, the Alarm phase, provides the initial response to stress, also referred to as the ‘fight or flight response’, ii) phase 2, the Resistance phase, demonstrates the body’s reaction/adaptation to the imposed stressors, whereby resources are focused to resist the elevated stress, and iii) phase 3, the Exhaustion phase, which can occur if the stress has continued for a prolonged duration, presents a loss in the body’s ability to resist the stress, and can ultimately be severely detrimental, possibly even causing death. It could be postulated that the changes in eHsp72 concentration in response to the initial increase in LPS translocation can offer a protective effect and may aid the reduction/prevention of heat-related illness associated with elevated LPS concentration. However, if the increase in LPS concentration is too large, the eHsp72

response may be insufficient and therefore unable to offer continuous protection against the imposed stress.



**Figure 2.4.** The General Adaptation Syndrome [Adapted from Selye (1946)].

The protective role of eHsp72 against elevated LPS concentration may occur through i) enhanced endotoxin tolerance (Aneja *et al.*, 2006), and ii) enhanced epithelial barrier function (Moseley *et al.*, 1994; Musch *et al.*, 1999). Firstly, endotoxin tolerance, a reduced capacity of the host to respond to LPS activation following exposure to relatively low LPS concentration (Fan and Cook, 2004), can have a protective effect and increase resistance to tissue injuries and mortality in models including infected thermal injury (He *et al.*, 1992), and hepatic ischemia/reperfusion (Heemann *et al.*, 2000). It has been shown that eHsp72 reduces the increase in pro-inflammatory cytokine release following LPS exposure, and subsequently leads to an increased level of tolerance against LPS (Aneja *et al.*, 2006). Aneja *et al.* (2006) stated that a higher eHsp72 concentration can increase tolerance to LPS, which can in turn reduce the risk of heat-related illness. Furthermore, it was demonstrated that monocytes treated with eHsp70 showed a greater reduction in the release of pro-inflammatory cytokines in response to LPS stimulation in comparison to non-treated cells (Aneja *et al.*, 2006). Cultured monocytes were treated with 0.03  $\mu\text{g/ml}$  eHSP70,

incubated for 18 h, then washed and stimulated with LPS. These *in vitro* findings demonstrate a protective role of eHsp70 against LPS through an enhanced level of tolerance. A greater eHsp72 concentration coincided with greater LPS tolerance, and as exercise heat-stress has been previously shown to induce a significant increase in eHsp72 concentration (Amorim *et al.*, 2008; Gibson *et al.*, 2014; Whitham *et al.*, 2007), it is possible that trained individuals undertaking such exercise will portray a greater degree of tolerance. Secondly, it is possible that increased eHsp72 concentration may serve to reduce LPS translocation by enhancing intestinal epithelial barrier function (Moseley *et al.*, 1994; Musch *et al.*, 1999). As stated in section 2.3.1, damage to the intestinal epithelial barrier can subsequently lead to a greater degree of LPS translocation into the circulatory system (Berkes *et al.*, 2003; Van Wijck *et al.*, 2011). Musch *et al.* (1999) demonstrated a protective role of eHsp72 upon epithelial barrier function against oxidant-induced stress, and further research has previously demonstrated that GI barrier protection is commonly linked to the increased eHsp72 concentration (Tao *et al.*, 2006; Wischmeyer *et al.*, 1997; Wischmeyer *et al.*, 2001). These findings indicate the importance of increased eHsp72 concentration on GI barrier function, which is essential for the reduction of LPS translocation (Lambert, 2004). Further research is required to further understand the relationship between eHsp72 and LPS concentration, and should focus on increasing the concentration of eHsp72 to protect against the intestinal damage that could lead to substantial LPS translocation. In addition, it is important to note that the aforementioned *in vitro* studies are less directly comparable to the changes that occur in plasma eHsp72, and thus whilst these changes give a clear indication of the potential effect that may occur, it is still essential to undertake further research *in vivo*, in order to fully understand the effect of LPS concentration upon the eHsp72 response.

## **2.4 Strategies to offset the endotoxemia response**

As demonstrated in section 2.4.1, GI distress caused by increased intestinal permeability can commonly occur during ultra-endurance exercise, particularly in the heat (Brock-Utne *et al.*, 1988; Jeukendrup *et al.*, 2000). Potential nutritional interventions have been explored, focusing on maintaining GI barrier integrity to prevent (though likely limit) LPS translocation during exercise heat-stress.

Bovine colostrum, the first milk produced after calving, is rich in nutrients and bioactive components (Playford *et al.*, 2000), and although supplementation has been shown to improve GI barrier integrity following heat-stress in rats (Prosser *et al.*, 2004), findings regarding the effect in humans have been equivocal. Marchbank *et al.* (2011) found an ~80% reduction in the rise in exercise-induced permeability following colostrum supplementation when compared to a placebo trial, whereas Morrison *et al.* (2014) found no physiological or performance benefits following 7 d supplementation. Ascorbic acid supplementation has been shown to abolish the increase in LPS both pre- and post-exercise (cycling to volitional exhaustion), however this alteration did not appear to influence exercise performance and lacks external validity to running based ultra-endurance events. Carbohydrate ingestion has demonstrated positive preventative effects (Jeukendrup, 2014; Oliveira *et al.*, 2014) but has also been shown to induce GI discomfort (de Oliveira and Burini, 2011). Currently it is difficult to identify the most effective *in vivo* method to reduce GI permeability and discomfort during exercise, and indicates the importance of robust empirically informed methods to prevent gut related negative effects during exercise heat-stress.

#### 2.4.1 Probiotic supplementation

Dietary probiotic supplementation has been shown to benefit individuals suffering from diseases affecting the GI tract, due to their proposed positive effects upon the gut and gut barrier integrity. Probiotic supplementation in animal models has demonstrated an improvement in gut integrity (Zareie *et al.*, 2006), and research into human clinical populations has often exhibited positive health effects of supplementation. Reduction in GI discomfort and GI side effects in individuals suffering from illnesses such as chronic fatigue syndrome (Lakhan and Kirchgessner, 2010), diarrhoea (Brigidi *et al.*, 2001; Chapman *et al.*, 2011), irritable bowel disease (Jonkers and Stockbrügger, 2003) and lactose intolerance (de Vrese *et al.*, 2001), have been found following probiotic supplementation.

There are a number of mechanisms by which probiotics exert these beneficial actions: i) through inhibiting the overgrowth of pathogenic bacteria (Vanderhoof *et*

*al.*, 1998), ii) by increasing the secretion of mucin (Otte and Podolsky, 2004), iii) by competing with pathogenic bacteria for binding sites on mucins and/or epithelial cells (Mack *et al.*, 2003), iv) through enhancing the stability of tight junctions between epithelial cells (Seth *et al.*, 2008) and v) through the activation of heat shock factor-1 (HSF-1), an activator responsible for the transcription of heat shock genes, leading to elevated Hsp72 concentration (Zuhl *et al.*, 2014). All of the above mechanisms can offer a greater understanding regarding the role of probiotic supplementation on GI health.

As stated in section 2.3.2, strenuous exercise can lead to increased epithelial tight junction permeability, which can ultimately allow increased LPS leakage into the circulation (Berkes *et al.*, 2003), leading to GI discomfort. Research investigating the role of probiotics during athletic performance has received little attention, however it is sensible to predict that by utilising probiotic supplementation to strengthen tight junction stability (Seth *et al.*, 2008), LPS translocation and GI discomfort during performance may be reduced.

Kekkonen *et al.* (2007) demonstrated a reduction in the duration of GI symptoms suffered by athletes during training and following a marathon after a three-month probiotic supplementation programme. This reduction in length of GI symptoms could be extremely beneficial for recovery between consecutive exercise bouts, which may be particularly important for multi-day events, such as the MDS, that require athletes to perform on successive days. In addition, Lamprecht *et al.* (2012) demonstrated the beneficial effects of a 14 wk supplementation period [ $10^{10}$  colony forming units (CFU) per day] on intestinal permeability in athletes at rest, which led to a 20% decrease in Zonulin concentration. Zonulin, a protein released from the liver and intestinal epithelial cells, is a main modulator of intercellular tight junctions (Fasano, 2011), of which increased concentration relates to changes in tight junction competency and GI permeability. The 14 wk supplementation programme implemented by Lamprecht *et al.* (2012) was evidently sufficient to initiate a reduction in resting GI permeability, however post-exercise GI permeability was not recorded, and thus conclusions regarding the effect of probiotic supplementation upon exercise-induced GI damage cannot be determined. Further research is therefore required to investigate the effect of probiotic supplementation on exercise-

induced GI damage, potentially through the implementation of longer duration exercise in hotter conditions.

Shing *et al.* (2014) demonstrated an improvement in running performance by 16% in a time to fatigue run at 80% ventilatory threshold (35°C, 40% RH) after 4 wk of probiotic supplementation in comparison with a placebo. Exercise induced a significant increase in LPS concentration from baseline in both conditions, however the pre- and post- exercise values were lower [although not statistically significant – Cohen’s effect size: pre: 0.70, post: 1.24 (Cohen, 1988)] in the probiotic compared to the placebo group. These findings indicated that probiotics may perhaps exert small to large effects on GI integrity and LPS translocation that may result in improvements upon exercise performance in the heat.

These studies (Kekkonen *et al.*, 2007; Lamprecht *et al.*, 2012; Shing *et al.*, 2014) demonstrate the role of probiotic supplementation on the GI response to exercise through exerting beneficial effects upon the intestinal tight junctions. A further mechanism of interest is the induction of eHsp72 to protect against GI damage. Research has shown that probiotic supplementation may enhance eHsp72 upregulation (Petrof *et al.*, 2004; Tao *et al.*, 2006). Novel research from Tao *et al.* (2006) investigated the effect of the probiotic strain *Lactobacillus* GG on Hsp72 expression in intestinal epithelial cells *in vitro*. Hsp72 has been shown to play a key cytoprotective role against damage to preserve epithelial tight junction and barrier function (Musch *et al.*, 1999), and thus it was hypothesised that a key mechanism of probiotic action is the ability to induce cytoprotective Hsps, such as Hsp72. *Lactobacillus* GG exposure led to the induction of eHsp72, which indicated that the cytoprotective role of probiotics may occur through increased eHsp72 concentration. Tao *et al.* (2006) also discovered a reduction/loss of most protective effects when Hsp72 was abolished from the cells, indicating the importance of this increased concentration for protection against epithelial cell injury.

In addition to probiotics, the amino acid glutamine (GLn), thought to be the primary fuel source for intestinal cells (Irvin and Heuberger, 2015), plays a key role in intestinal function, structure and metabolism (Akobeng *et al.*, 2000). Similar to the effects of probiotics, oral GLn supplementation has been shown to enhance intestinal

barrier function by decreasing intestinal permeability in both animal (dos Santos *et al.*, 2010) and human (Zuhl *et al.*, 2014) models, with a key protective mechanism thought to be through the induction of Hsp72 (Wischmeyer, 2002; Zuhl *et al.*, 2015). Wischmeyer *et al.* (2001) demonstrated that a single dose of intravenous GLn caused a rapid and significant increase in Hsp72 expression in a rat model. Additionally, endotoxin-treated animals given GLn exhibited a substantial increase in tissue Hsp expression and a decrease in end-organ damage. These findings indicated GLn to be a clinically viable enhancer of Hsp expression. Zuhl *et al.* (2015) supported these findings and demonstrated that oral GLn supplementation attenuated an exercise-induced rise in intestinal permeability, and subsequently increased HSP70 induction. Furthermore, Zuhl *et al.* (2014) found that the exercise-induced increase in intestinal permeability caused by 60 min running at 70%  $\dot{V}O_{2\max}$  was completely ameliorated in the GLn trial. This protective effect was thought to be associated with HSF-1 activation, which leads to protein stabilisation and a reduction in intestinal permeability, and thus a subsequent increase in HSP70 expression.

Evidently, both probiotic and GLn supplementation have demonstrated protective roles against GI discomfort, damage and dysfunction. Research investigating the relationship between probiotic supplementation, gut health and the eHsp72 response in humans has received little attention, and the effect during ultra-endurance exercise is unknown. The combination of exercise-induced heat-stress and exercise-induced gut damage may lead to an increase in the concentration of cytoprotective eHsp72; which may lead to a greater level of protection, and thus reduce the risk of GI related illness. In addition, the added benefits of GLn combined with probiotic supplementation could lead to greater improvements in gut barrier integrity and a reduction in intestinal permeability through increased Hsp72 concentration.

## **2.5 Overall summary**

It is evident that endurance and ultra-endurance running in the heat can often induce an increase in GI permeability and subsequent LPS translocation, leading to severe discomfort for the individual (Berkes *et al.*, 2003; Brock-Utne *et al.*, 1988; Jeukendrup *et al.*, 2000). The resultant level of stress from such exercise can cause



an increase in the concentration of eHsp72 as a method of cytoprotection (Lee *et al.*, 2006; Moseley, 1997). This elevated eHsp72 concentration may reduce GI barrier damage (Musch *et al.*, 1999), however, the role of eHsp72 in response to exercise-induced endotoxemia requires further investigation.

In order to enhance protection against such side effects, nutritional interventions can perhaps be employed. Both probiotic and glutamine supplementation have been shown to offer beneficial effects through the strengthening of tight junctions between epithelial cells (Seth *et al.*, 2008) and via increased eHsp72 concentration (Zuhl *et al.*, 2014). However, the effect of these interventions upon the GI and heat shock responses to ultramarathon performance has not yet been investigated, and thus further research is required, which could provide novel knowledge and understanding indicating a method by which stress can be reduced during ultramarathon events.

## **2.6 Aims and hypothesis**

To investigate the effects of 12 wk probiotic supplementation on LPS translocation and the resulting eHsp72 response following a multi-day ultra-endurance event in extreme heat.

1. Probiotic (PRO) and probiotic + glutamine (PGLn) supplementation will increase eHsp72 concentration from baseline to pre-race in comparison to CON.
2. Post-race, eHsp72 concentration will be significantly increased in all groups.
3. PRO and PGLn will demonstrate a significantly greater eHsp72 concentration in comparison to CON.
4. LPS concentration following the MDS will show the greatest increase in the CON group compared to PRO and PGLn.

## **2.7 Novelty of Research Project**

To reiterate the novelty of this research project, it has been shown that previous research has investigated the eHsp72 response to ultra-endurance events, and has demonstrated the role of PRO upon LPS concentration. However, this study is the first to collect data from a large sample following the MDS, a race that takes place in a logistically challenging location and environment. Additionally, this novel study has, for the first time, investigated the role of PRO and PGLn upon both eHsp72 and LPS concentration over a 12 wk supplementation period, following the MDS, and 7 d post-race to investigate the duration of the effect. This study therefore aims to address the gap in the knowledge, whereby understanding the effect of PRO upon the eHsp72 and LPS response during ultra-endurance events could have a significant impact upon an individual and the GI response to such exercise.

## **CHAPTER 3: Experimental Chapter - Chronic Probiotic Supplementation and its Effects on eHsp72 and LPS Concentration Following a Desert-Based Ultramarathon**

### **3.1 Introduction**

Exercise in extreme environmental conditions can place severe physiological stress on the human body (Nielsen and Nybo, 2003; Walsh and Whitham, 2006; Nybo, 2008). Such stress (see section 2.3.1) can elicit an increase in eHsp72 concentration (Marshall *et al.*, 2006; Whitham *et al.*, 2007; Périard *et al.*, 2012; Ruell *et al.*, 2014), in order to offer a protective role through preparing the immune system for the associated exercise-induced stressors (Johnson and Fleshner, 2006).

Prolonged exercise in such environments can result in an increased incidence of GI dysfunction and discomfort, including symptoms of nausea, stomach cramps and bloating (Jeukendrup *et al.*, 2000; Hoffman and Fogard, 2011); with the risk of further serious conditions, including haemorrhagic gastritis, hematochezia and ischemic bowel (Strid and Simrén, 2005). Such GI dysfunction can be attributed to the leakage of LPS from the GI mucosa into the systemic circulation (Berkes *et al.*, 2003; Yeh *et al.*, 2013), often detrimentally affecting performance and ‘overall’ health of the individual or athlete (Ter Steege and Kolkman, 2012).

To acquiesce such exercise-induced GI damage, nutritional interventions have been trialled to improve overall gut health (Jeukendrup, 2014; Morrison *et al.*, 2014; Oliveira *et al.*, 2014). Clinical probiotic supplementation has been shown to improve gut health and offer protection/treatment relative to various diseases, including inflammatory bowel disease (Shadnouch *et al.*, 2015), Crohn’s disease (Guslandi *et al.*, 2000; Gupta *et al.*, 2000) and ulcerative colitis (Cui *et al.*, 2004; Matthes *et al.*, 2010). Initial research suggests potential beneficial health effects, particularly attributed to gut health, of probiotic supplementation in trained individuals [see section 2.4.1; (Kekkonen *et al.*, 2007; Gleeson *et al.*, 2011; Pyne *et al.*, 2015)]. Supplementation of both PRO (Tao *et al.*, 2006; Petrof *et al.*, 2004) and GLn

(Wischmeyer, 2002) have demonstrated the ability to induce eHsp72, which plays a crucial indirect role in maintaining gut epithelial barrier integrity (Amorim and Moseley, 2010). Thus, the enhancement in GI health induced by PRO or a combination of PRO and GLn (PGLn) supplementation (mechanisms described in section 2.4.1), alongside the potential role of probiotics upon eHsp72 induction could, at least hypothetically, reduce the detrimental effects of LPS translocation in athletes during ultra-endurance exercise

### 3.1.1 Aims and Hypothesis

The aim of this study is to investigate the effect of chronic (12 wk) probiotic supplementation on LPS translocation, GI discomfort and the eHsp72 response to ultra endurance running in extreme heat.

The experimental hypotheses were as follows:

1. PRO and PGLn supplementation will increase eHsp72 concentration from baseline to pre-race in comparison to CON.
2. Post-race eHsp72 concentration will be significantly increased in all groups.
3. PRO and PGLn will demonstrate a significantly greater eHsp72 concentration in comparison to CON.
4. LPS concentration following the MDS will show the greatest increase in the CON group compared to PRO and PGLn.

## 3.2 Methods

### 3.2.1 Participants

Thirty-two competitors taking part in the MDS 2015 (6 female, age 41; range 23-53 years, height  $1.75 \pm 0.08$  m, body mass  $76.89 \pm 2.04$  kg) were recruited for this study. Prior to commencement of the study, participants were provided with a detailed information sheet (Appendix A), outlining the purpose, procedures and risks of the study, and were made aware that they were free to withdraw from the study at any time. All participants provided written informed consent (Appendix B), were deemed healthy and able to take part (Appendix C), and all participants verbally confirmed that they were not currently undertaking any probiotic or glutamine supplementation regime. All procedures were approved by the Anglia Ruskin University Ethics Committee, and conformed to the Declaration of Helsinki.

### 3.2.2 Probiotic supplementation

Following a randomised, independent measures design, using simple randomization, participants were assigned to one of three experimental conditions: probiotic capsules (PRO), probiotic + glutamine powder (PGLn), or control (CON). Participants were instructed as to the requirements for their specific group and were required to follow these instructions to ensure compliance to the study intervention; daily adherence to the intervention was self-reported at 100% for all groups. Due to the nature of the study, blinding of groups was not possible, however, the PGLn group were unaware of the addition of GLn to their probiotic supplement.

*PRO*: were required to consume one capsule (BioAcidophilus Forte, UK) per day for the duration of the 12 wk intervention period; capsules contained *Lactoballicus acidophilus* (100 mg), Fructooligosaccharides (60 mg), *Bifidobacterium bifidum* (22.2 mg) and *Bifidobacterium lactis* providing a total of 30 billion viable proprietary organisms (see Appendix D for full ingredients).

*PGLn*: were required to consume 5 g powder (GI Complex, UK) per day, mixed well in water or food. The key probiotic strains in the PGLn were *Lactobacillus*

*acidophilus* (40.5 mg), *Lactobacillus salivarius* (25 mg), *Bifidobacterium bifidum* (3.5 mg) and *Bifidobacterium lactis*, providing a total of 10 billion viable organisms; each 5 g dose also contained 0.9 g L-Glutamine (see Appendix E for full details).

*CON*: were required to continue with their normal diet, and were to refrain from any substantial changes and supplementation regime.

All supplements were donated to the research team by BioCare, UK, in order for use in this research study. Dosages were selected based on the recommended dosage from BioCare, UK, in order to investigate an ecologically valid supplementation regime.

### 3.2.3 Study overview

Data was collected at four time points across the duration of the study, which consisted of three laboratory visits and one field-based data collection point. Data was collected on two occasions prior to the race: i) 12 wk (visit 1; baseline) and ii) 7 d (visit 2; pre-race) prior to departure for the MDS, and two further occasions post-race: iii) 6 - 8 h post-race (post-race data collection) and iv) 7 d post-race (visit 3).

*Visits 1 and 2*: Participants arrived at the environmentally controlled laboratory (18°C, 35% RH) in a fasted state (minimum 4 h fasted) with consumption of alcohol and caffeine, and undertaking of physical activity 24 h prior to each visit forbidden, with apparent adherence confirmed at 100% for all participants. Upon arrival, participants rested in a semi supine position for measures of resting heart rate [(HR), Polar, FS1, UK], followed by a venous blood sample via venepuncture from the antecubital fossa for analysis of eHsp72 and LPS (as detailed further in section 3.2.7). Body composition was subsequently measured via skinfolds (section 3.2.5), followed by the completion of a  $\dot{V}O_{2\max}$  test (section 3.2.6; see Figure 3.1 for experimental schematic).

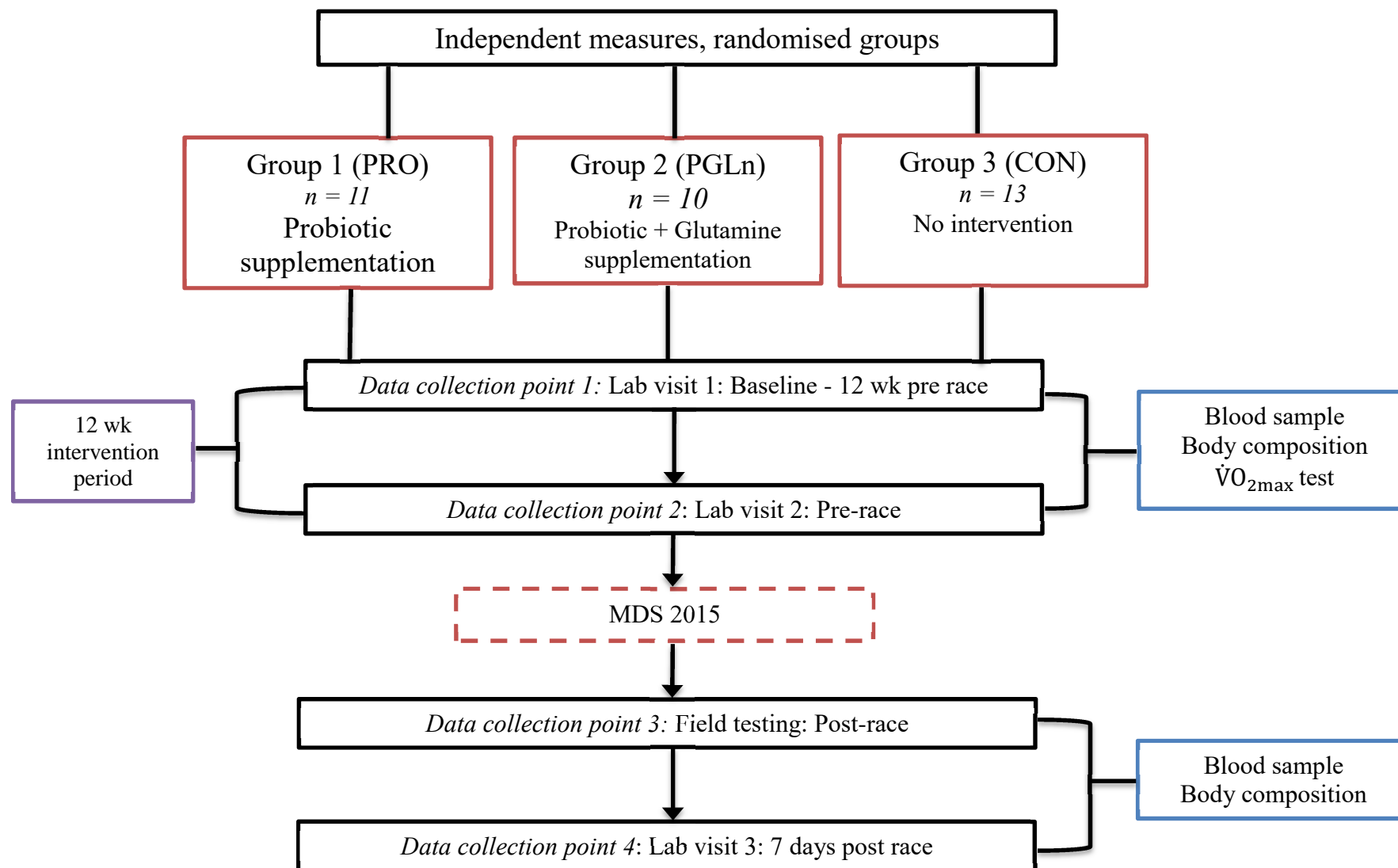
*Post-race data collection*: Upon completion of the race (full race details provided in section 3.2.4), participants boarded coaches and were taken from the Desert back to

the city of Ouarzazate, for post-race data collection. A team of experimenters measured body composition and collected post-race venous blood samples (in line with procedures outlined in sections 3.2.5 and 3.2.7, respectively); this data collection took place 6 - 8 h post-race completion.

*Visit 3:* Participants attended the sport science laboratories 7 d post-race, which involved the same experimental procedures as visits 1 and 2, but excluded the completion of a  $\dot{V}O_{2\max}$  test.

### 3.2.4 The Marathon des Sables

The MDS 2015 took place from 5<sup>th</sup> – 11<sup>th</sup> April, and covered a total distance of 249.4 km across the Sahara Desert, Morocco (average temperature ~ 38°C), over 7 days. Each stage started at 0900 h and consisted of the following distances completed in one day unless indicated otherwise: stage 1 (36.2 km), 2 (31.1 km), 3 (36.7 km), 4 (91.7 km; two day stage), 5 (42.2 km) and 6 (11.5 km). The MDS required competitors to be self sufficient, meaning they were to carry their own food (minimum 1500 kcal per day), equipment, and sleeping materials for the duration of the race. Water was rationed to ~ 9.0 – 10.5 L/day per competitor, dependent on the distance of the stage. At the end of each stage, participants were required to complete a GI symptoms questionnaire [(Appendix F) adapted by Roberts, J.D. and Roberts, M.G. (2013) from Lipski (1998)] independently without direct experimenter oversight, detailing any general and/or specific GI discomfort experienced throughout the entire stage. Participants were required to complete this questionnaire within 2 h of stage completion; adherence was reported at 100% across the duration of the race.



**Figure 3.1.** Experimental schematic outlining the experimental study design and data collection time points.



### 3.2.5 Body composition

A seven-site skinfold measurement using skinfold calipers (Harpenden, Cranlea, UK) was conducted to calculate body fat percentage using the appropriate American College of Sports Medicine (ACSM) equation (ACSM, 2013). Two measurements were taken from each site by the same qualified experimenter for all participants, in order to obtain a mean value and increase reliability of results.

### 3.2.6 $\dot{V}O_{2\max}$

During visits 1 and 2, participants were required to undertake a graded exercise test to maximal exhaustion on a motorized treadmill (Pulsar, HP Cosmos, UK) for the measurement of  $\dot{V}O_{2\max}$ . Participants were fitted with a HR monitor (Polar, FS1, UK) and suitably sized metalyser mask (Cortex, UK). After a 5 min self-paced warm up, participants exercised to volitional exhaustion following a standardized incremental protocol from the British Association of Sport and Exercise Sciences [BASES; (Winter *et al.*, 2006)]. Starting speed was selected based on the individuals' preferred running speed. Speed was increased by 1 km/h<sup>-1</sup> every 2 min, after 4 stages (8 min) speed remained constant and treadmill incline increased by 1% every 2 min until volitional exhaustion (Winter *et al.*, 2006). Online breath-by-breath analysis (Metalyser 3B, Cortex, UK) was used to determine  $\dot{V}O_{2\max}$ , and measures of HR and ratings of perceived exertion (RPE) were recorded every 2 min to be used as secondary criteria. The  $\dot{V}O_{2\max}$  was considered as the highest  $\dot{V}O_2$  obtained in any 10 s period, and in line with end point criteria guidelines of the ACSM; which required participants to meet a plateau in  $\dot{V}O_2$ , plus 2 of the 3 following criteria: a failure of HR to increase with increasing exercise intensity, respiratory exchange ratio (RER) of > 1.15, and RPE > 17 (ACSM, 2013).

### 3.2.7 Blood collection and analysis

Venous blood samples were collected at all four data collection time points. A 12 ml whole blood sample was drawn from the antecubital fossa via venepuncture (Safety blood collection set and holder, Vacuette®, Greiner Bio-One, UK), directly into

three separate Vacuette® tubes (Vacuette® Grenier Bio-One, UK) treated with K3 Ethylenediaminetetraacetic acid (EDTA) coagulant (3 x 4 mL tubes). Whole blood samples were centrifuged (EBA 200, Hettich, Germany) at 3000 rpm for 10 min for plasma separation, which was then pipetted into 1.5 ml Eppendorf tubes (Fisher Scientific, UK) and stored at - 80°C until analysed for eHsp72 and LPS using a commercially available high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits.

### 3.2.7.1 Extracellular heat shock protein 72

A commercially available ELISA kit (HSP70 high sensitivity ELISA kit, Enzo Life Sciences, Exeter, UK) was used to determine eHsp72 concentration in plasma samples (see appendix G for raw plate layouts and data). Determination of eHsp72 was performed according to the manufacturers guidelines. Briefly, incubation of the 96-well kit was performed on an incubital shaker (Heidolph Titramax 1000, Fischer Scientific, UK) at 500 rpm, and read by a plate reader (VICTOR™ X, Perkin Elmer, UK) using absorption at 450 nm. A graph for linearity between known samples concentration and optical density was used to ensure accuracy of sample data (Appendix H). Translation of raw plate reader data to eHsp72 units ( $\text{nm/ml}^{-1}$ ) was performed using a linear trendline and equation (see Appendix H). To eliminate intra-assay variation, samples were measured in duplicate where possible. Intra-assay variability was 9.93%; which was in line with previous work in the field: Campisi *et al.* (2003): < 10%; Gibson *et al.* (2014): 10.5%; Périard *et al.* (2012): 5%; Walsh *et al.* (2001): < 10%; Whitham *et al.* (2006): 6.3%.

### 3.2.7.2 Lipopolysaccharides

Changes in circulatory LPS concentration were determined using a commercially available ELISA kit (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, ThermoFisher Scientific, UK) following the manufacturers guidelines (see appendix G for raw plate layouts and data). Two additional dilutions were applied to add two extra calibration points at the lower end of the calibration curve, to increase accuracy of readings at the lower end of the curve. Briefly, incubation of the 96-well kit was performed on an incubital shaker (Heidolph Titramax 1000, Fischer Scientific, UK)

at 500 rpm, and read by a plate reader (VICTOR™ X, Perkin Elmer, UK) using absorption at 405 nm. Endotoxin concentration was determined using a standard curve plotting the blank-corrected absorbance for each standard versus its concentration in EU/mL (Appendix I). The co-efficient of determination was > 0.98 for all assays. To eliminate intra-assay variation, samples were measured in duplicate where possible. Intra-assay variability was 3.7%; previous LPS research has failed to report this data, and thus, based on the guidelines of Reed *et al.* (2002), an intra-assay variability of < 20% is commonly accepted as reliable.

### 3.2.8 Statistical analysis

All data were analysed using IBM SPSS Statistics for Macintosh, Version 21 (IBM Corp, Armonk, NY). Quantile-quantile (Q-Q) plots were used to assess the normality of distribution for all variables. All data is presented as mean  $\pm$  standard deviation (SD) when normally distributed, for data that violated the normality assumption the median and range is reported, statistical significance was accepted at  $p < 0.05$ . In line with previous Hsp72 research (Morton *et al.*, 2006; 2007; 2008; Tuttle *et al.*, 2015) linear mixed model analysis was used to identify the effect of time, group, and group x time on the dependent variables. Data were analysed for differences between genders, where a difference was found between genders, females and males data are reported separately, where no difference was found between males and females, data were collapsed and analysed as a whole in line with the principal of parsimony. In accordance with previous literature, eHsp72 (Gibson *et al.*, 2014; Morton *et al.*, 2007; Peart *et al.*, 2011; Sandström *et al.*, 2008; Suzuki *et al.*, 2006; Taylor *et al.*, 2010a; Taylor *et al.*, 2010b) and LPS (Costa *et al.*, 2014; Gill *et al.*, 2015; Olesen *et al.*, 2015) concentrations were expressed as a percentage change from baseline, to account for high individual variance in baseline values and responses.

### 3.2.9 Research team

As stated, this research was conducted in collaboration with the Sport and Exercise Science Research Group at Anglia Ruskin University, Cambridge. I (Hannah Marshall), was present at three of the four data collection blocks, and aided with all lab-based data collection. Due to funding issues, it was not possible to attend data

collection point 3 (field testing 6-8 h post-race, Morocco). Two members of the research team, [Craig Sucking (Ph.D. student, Anglia Ruskin University) and myself], ran the ELISAs for analysis of eHps2 concentration, whilst LPS ELISAs were ran separately at Anglia Ruskin University, with raw data being sent to myself for analysis.

### 3.3 Results

#### 3.3.1 Group characteristics

Anthropometrical data is displayed in Table 3.1. Linear mixed model analysis revealed that there was a significant effect of time on  $\dot{V}O_{2\max}$  ( $F_{2,29} = 17.537$ ,  $p < 0.001$ ). Pre-race mean  $\dot{V}O_{2\max}$  was significantly greater than baseline mean  $\dot{V}O_{2\max}$  by  $3.92 \text{ ml.kg.min}^{-1}$  (95% CI:  $2.003 - 5.845 \text{ ml.kg.min}^{-1}$ ). There was no significant effect of group ( $F_{2,27} = 0.963$ ,  $p = 0.393$ ) or group x time ( $F_{2,27} = 0.077$ ,  $p = 0.926$ ) on  $\dot{V}O_{2\max}$ . Additionally, there was a significant effect of time on body mass, with a significant reduction observed from  $76.89 \pm 2.04 \text{ kg}$  to  $75.27 \pm 2.04 \text{ kg}$  from baseline to pre-race, respectively ( $F_{1,28} = 18.712$ ,  $p < 0.001$ ). There was no significant group ( $F_{2,29} = 1.417$ ,  $p = 0.259$ ) or group x time ( $F_{2,28} = 2.828$ ,  $p = 0.076$ ) effect on body mass. There was a significant effect of gender on body fat % ( $F_{1,238} = 49.803$ ,  $p < 0.001$ ), which was shown to be significantly lower in male participants ( $18.43 \pm 0.36\%$ ) compared to females [ $(24.77 \pm 0.83\%)$ ; 95% CI:  $4.574 - 8.117\%$ ]. A significant gender x group effect ( $F_{2,238} = 13.842$ ,  $p < 0.001$ ) displayed that females in the CON group ( $31.25 \pm 1.06\%$ ) had a greater body fat % than females in the PRO ( $21.26 \pm 1.83\%$ ) and PGLn ( $21.63 \pm 1.29\%$ ) groups, no significant difference was shown between groups for male participants. There was no significant effect of gender x time ( $F_{3,238} = 0.037$ ,  $p = 0.990$ ) or gender x group x time ( $F_{6,238} = 0.673$ ,  $p = 0.671$ ) on body fat %. There was no significant effect of group ( $F_{2,31} = 0.294$ ,  $p = 0.747$ ), time ( $F_{1,31} = 0.438$ ,  $p = 0.513$ ) or group x time ( $F_{2,31} = 0.070$ ,  $p = 0.932$ ) on HR.

Two participants (both from the CON group) were disqualified from the race due to failing to complete a stage in the required cut-off time. Linear mixed model analysis

demonstrated a significant effect of group on overall MDS finishing time ( $F_{2,65} = 4.147, p = 0.020$ ). Sidak post-hoc tests demonstrated that the CON group completed the race in a significantly slower time ( $3043 \pm 151$  min) in comparison to PRO ( $2437 \pm 164$  min, 95% CI: 59.936 – 1152.400 min). Furthermore, following the removal of the two disqualified participants, the Linear mixed model analysis was re-run to assess any difference in results, and demonstrated a significant effect of group on finishing time ( $F_{2,58} = 4.023, p = 0.023$ ). Sidak post-hoc tests revealed similar results to the previous analysis, whereby the MDS finishing time of the CON group ( $3052 \pm 181$  min) was significantly slower than PRO ( $2249 \pm 242$  min, 95% CI: 60.272 – 1546.35 min). There was no significant difference between CON and PGLn ( $2534 \pm 183$  min), or PRO and PGLn. There was no significant effect of gender ( $F_{1,58} = 0.176, p = 0.676$ ) or gender x group ( $F_{2,58} = 3.132, p > 0.05$ ).

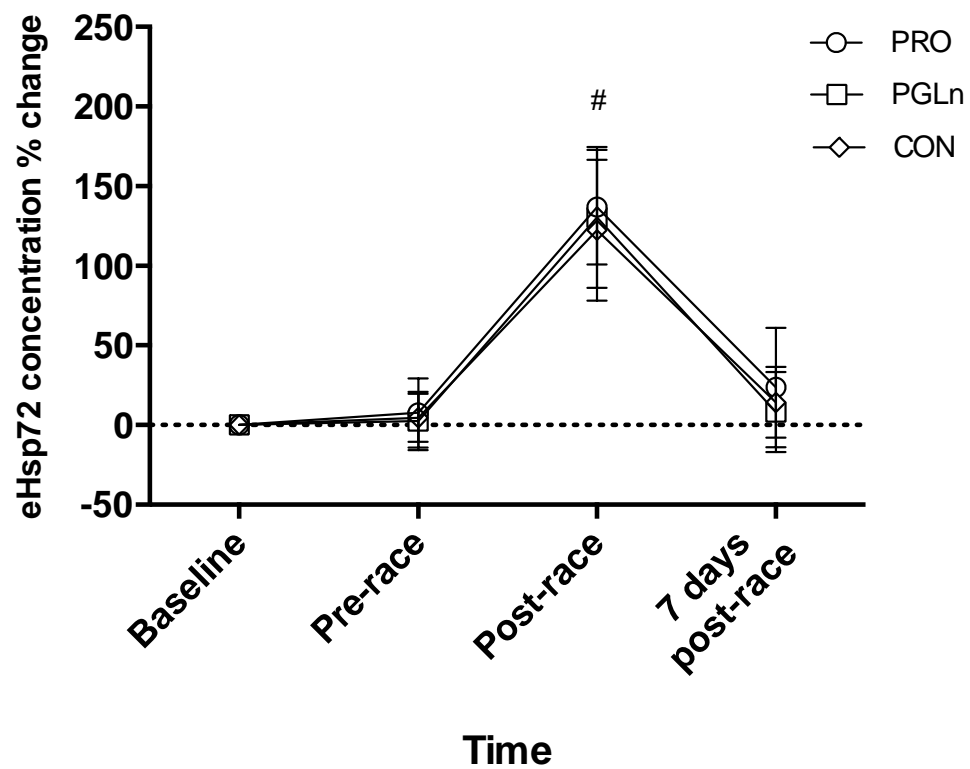
**Table 3.1.** Anthropometrical characteristics of age, height, mass, body fat percentage, and  $\dot{V}O_{2\max}$  data (wk 1 and wk 12) are reported as means  $\pm$  SD.

Group	Age (years)	Baseline				Pre-race			
		Resting HR (b.min <sup>-1</sup> )	Mass (kg)	Body fat %	$\dot{V}O_{2\max}$ (ml.kg.min <sup>-1</sup> )	Resting HR (b.min <sup>-1</sup> )	Mass (kg)	Body fat %	$\dot{V}O_{2\max}$ (ml.kg.min <sup>-1</sup> )
PRO	38 $\pm$ 8	52 $\pm$ 8	78.87 $\pm$ 6.93	20.32 $\pm$ 3.99	56.42 $\pm$ 8.26	51 $\pm$ 5	77.49 $\pm$ 6.52	18.76 $\pm$ 2.73	59.40 $\pm$ 6.33
PGLn	40 $\pm$ 8	55 $\pm$ 10	71.34 $\pm$ 12.92	19.74 $\pm$ 4.54	56.50 $\pm$ 5.95	55 $\pm$ 10	70.09 $\pm$ 12.31	19.07 $\pm$ 4.14	59.25 $\pm$ 6.92
CON	42 $\pm$ 9	54 $\pm$ 12	79.50 $\pm$ 14.07	22.72 $\pm$ 8.56	51.17 $\pm$ 12.68	54 $\pm$ 14	77.29 $\pm$ 12.92	22.80 $\pm$ 9.32	55.25 $\pm$ 11.96

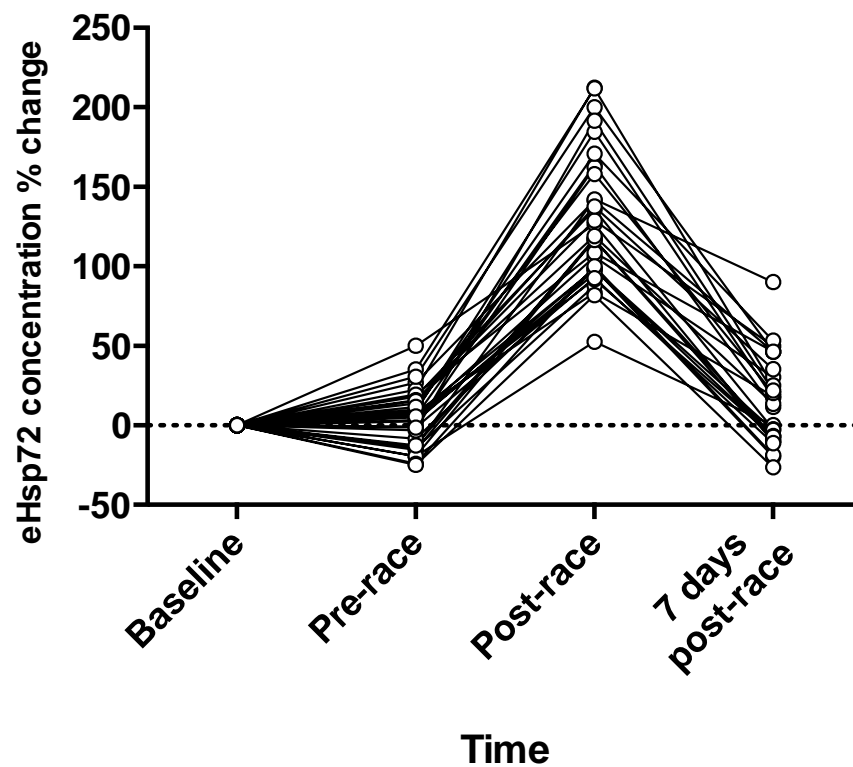
HR: Heart Rate; b.min<sup>-1</sup>: beats per minute; kg: kilograms;  $\dot{V}O_{2\max}$ : maximal volume of oxygen consumption; ml.kg.min<sup>-1</sup>: millilitres per kilogram per minute. PRO: Probiotic; PGLn: Probiotic + Glutamine; CON: Control.

### 3.3.2 eHsp72

Figure 3.2 illustrates overall mean eHsp72 concentration for PRO, PGLn and CON at baseline, pre-race, post-race and 7 d post-race. Linear mixed model analysis revealed that there was a significant effect of time on overall mean eHsp72 concentration ( $F_{3,88} = 162.698$ ,  $p < 0.001$ ). Overall mean eHsp72 concentration was significantly greater post-race by 124% (95% CI: 106.287 – 141.782%), 122% (95% CI: 103.927 – 139.781%) and 111% (95% CI: 93.373 – 130.545%) compared to baseline, pre-race and 7 d post-race, respectively (raw values are displayed in Table 3.2). There was no significant effect of group ( $F_{2,31} = 1.304$ ,  $p = 0.286$ ) or group x time ( $F_{6,88} = 0.662$ ,  $p = 0.680$ ) on eHsp72 concentration (Figure 3.3 shows overall individual responses, Figure 3.4 a-c shows individual eHsp72 responses within groups over time). There was no significant correlation between post-race eHsp72 concentration and MDS finishing time ( $r = 0.002$ ,  $p = 0.991$ ).

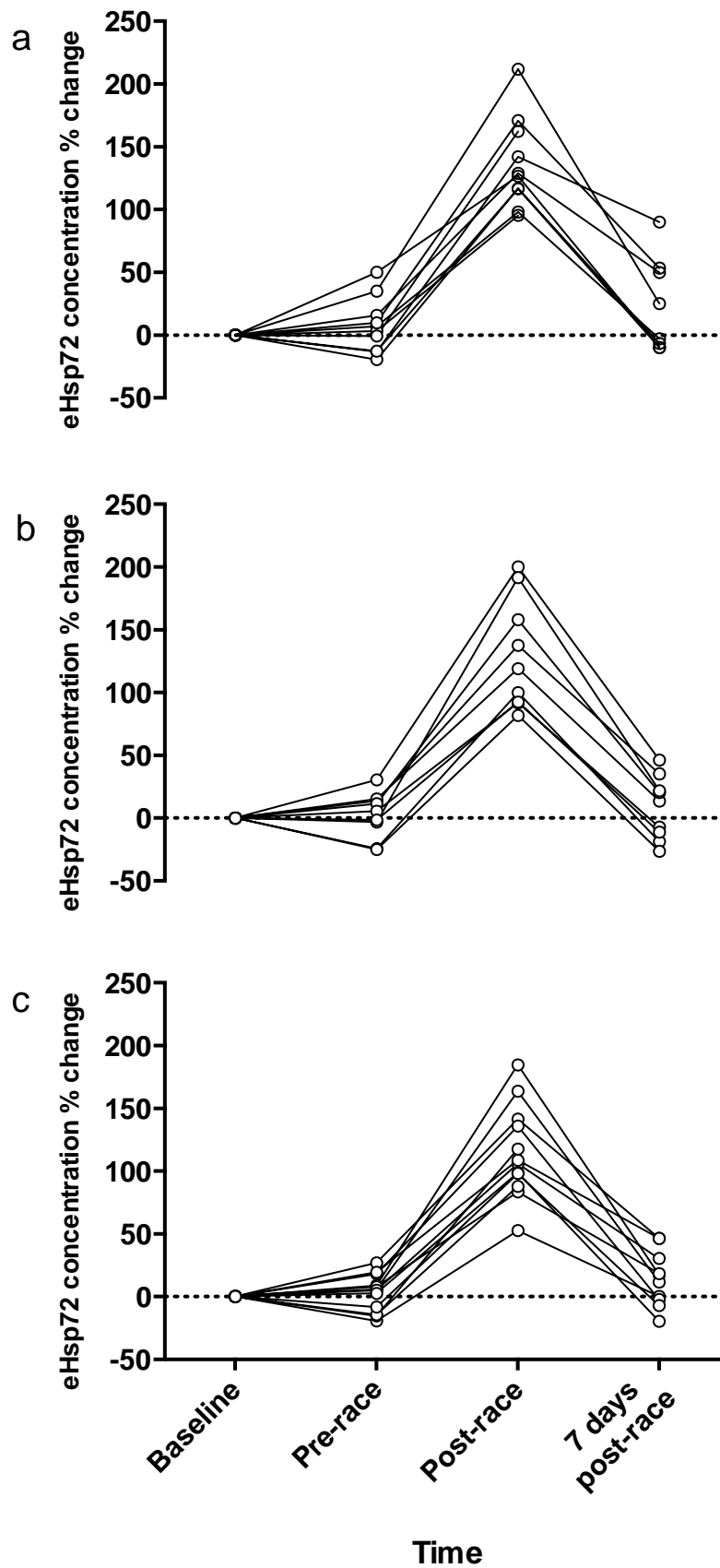


**Figure 3.2.** Mean ( $\pm$  SD) eHsp72 response between groups, as a % change from baseline. #Denotes significantly greater eHsp72 concentration post-race compared to all other time points ( $p < 0.05$ ).



**Figure 3.3.** Overall individual eHsp72 response over time as a % change from baseline.

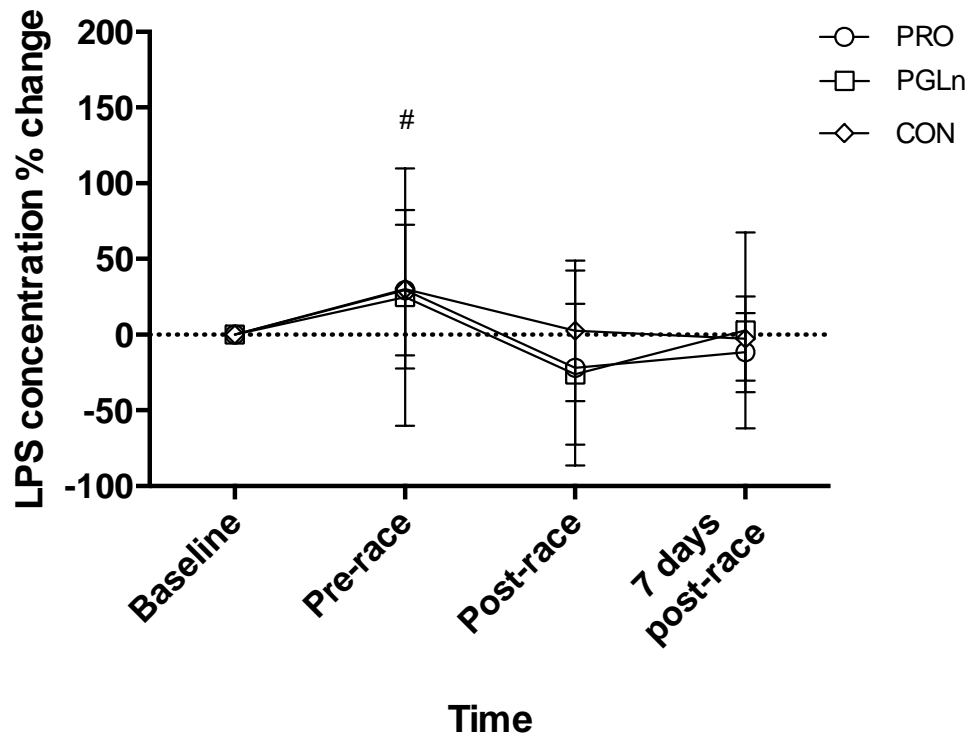




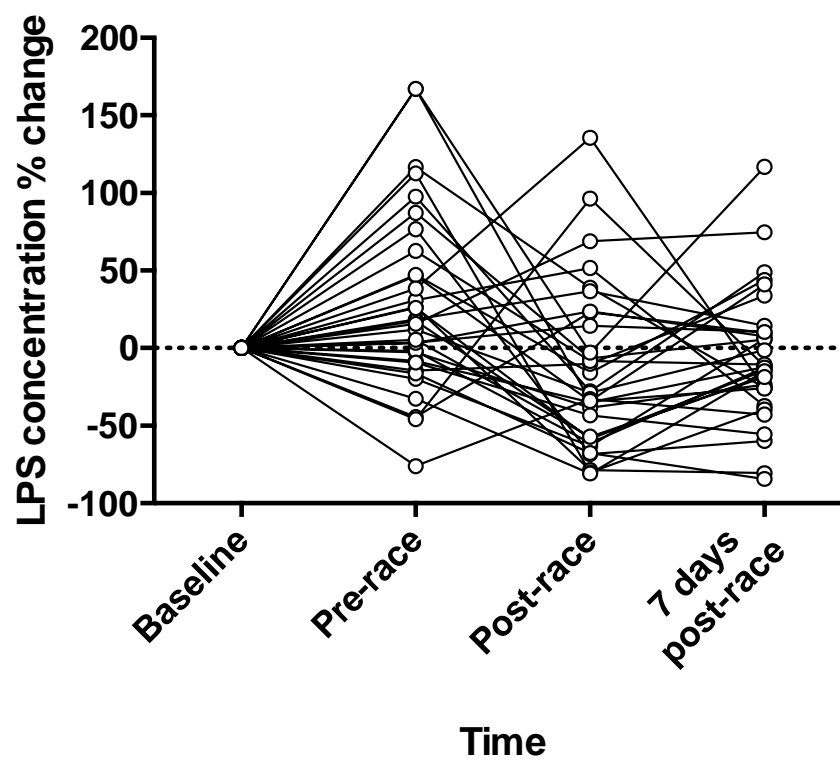
**Figure 3.4 a-c.** Individual eHsp72 responses within groups, as a % change from baseline. (a: PRO, b: PGLn, c: CON).

### 3.3.3 LPS Concentration

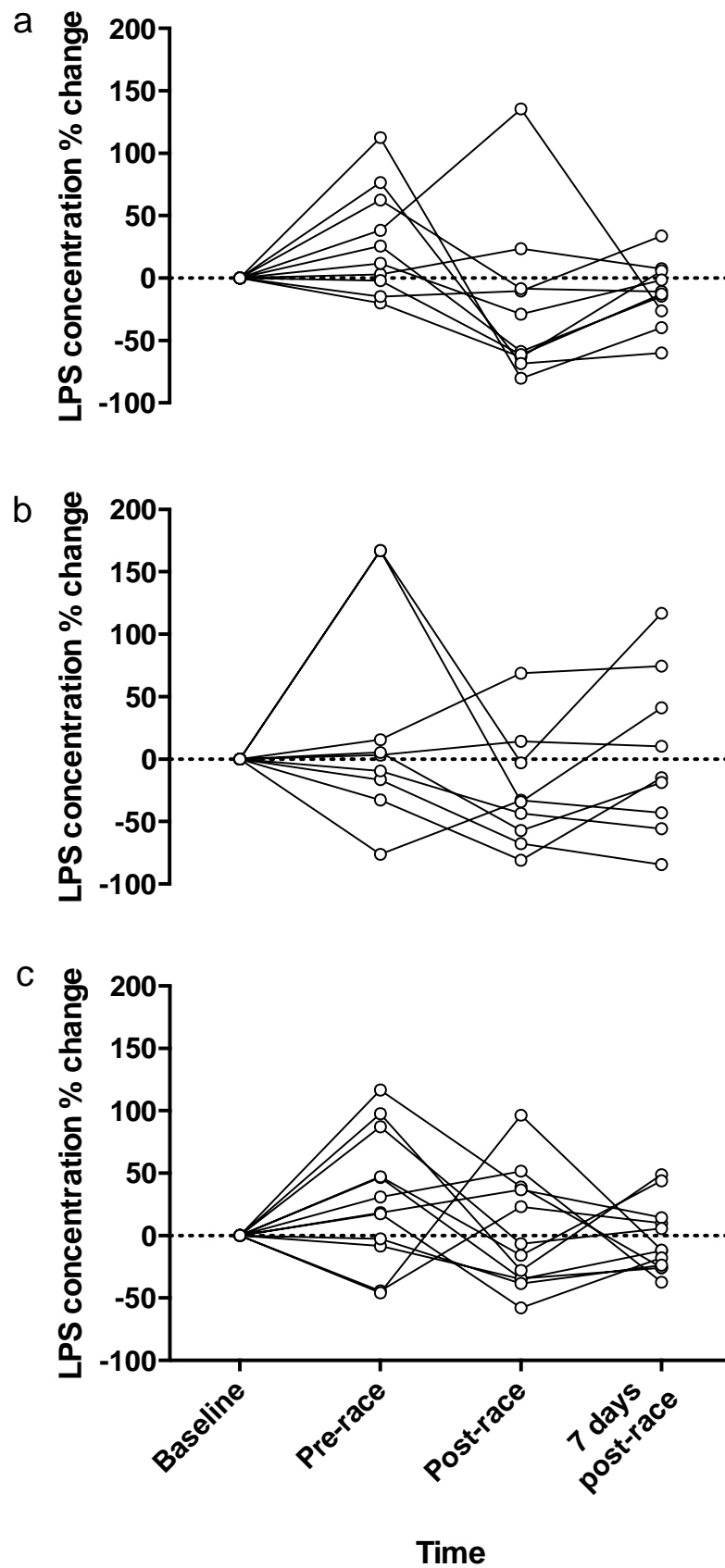
A significant effect of time was observed for overall mean LPS concentration ( $F_{3,91} = 5.668$ ,  $p < 0.001$ ). Pre-race LPS concentration was 31, 43 and 30% greater than baseline, post-race and 7 d post-race, respectively (see Figure 3.5 for overall group mean LPS concentration). There was no significant effect of group ( $F_{2,31} = 0.401$ ,  $p = 0.673$ ) or group x time ( $F_{6,91} = 0.583$ ,  $p = 0.743$ ) on LPS concentration (Figure 3.6 shows overall individual responses, Figure 3.7 a-c shows individual eHsp72 responses within groups over time, and raw data is displayed in Table 3.2). There was no significant correlation between post-race LPS concentration and MDS finishing time ( $r = 0.114$ ,  $p = 0.529$ ).



**Figure 3.5.** Mean ( $\pm$  SD) LPS response between groups, as a % change from baseline. #Denotes significantly greater LPS concentration at pre-race compared to all other time points ( $p < 0.05$ ).



**Figure 3.6.** Overall individual LPS responses, as a % change from baseline.



**Figure 3.7. a-c.** Individual LPS response within groups, as a % change from baseline. (a: PRO, b: PGLn, c: CON).

### 3.3.4 eHsp72 and LPS

A Pearson's correlation was used to determine the relationship between overall mean eHsp72 and LPS concentration at each data collection time point. A significant correlation was found between variables pre-race ( $r^2 = 0.428$ ,  $p = 0.013$ ), however no correlation was observed post-race ( $r^2 = 0.122$ ,  $p = 0.492$ ) or 7 d post-race ( $r^2 = 0.24$ ,  $p = 0.900$ ).

### 3.3.5 GI discomfort

Mild GI discomfort, including symptoms such as belching and nausea, was reported by 64, 56, and 58% of participants in PRO, PGLn, and CON groups, respectively throughout the entire race. There was no significant difference ( $p > 0.05$ ) in GI discomfort between groups.

**Table 3.2.** Mean ( $\pm$  SD) eHsp72 ( $\text{ng}\cdot\text{ml}^{-1}$ ) and LPS ( $\text{pg}\cdot\text{ml}^{-1}$ ,  $\text{EU}\cdot\text{ml}^{-1}$ ) concentrations between at each data collection time point.

Group	Baseline	Pre-race	6 hours post race	7 d post-race
<b>eHsp72 (<math>\text{ng}\cdot\text{ml}^{-1}</math>)</b>				
CON	$1.68 \pm 1.65$	$1.82 \pm 1.98$	$3.45 \pm 3.35$	$2.05 \pm 2.49$
PRO	$1.20 \pm 0.58$	$1.58 \pm 0.87$	$2.77 \pm 1.21$	$1.80 \pm 1.27$
PGLn	$1.77 \pm 2.51$	$1.03 \pm 0.45$	$2.20 \pm 0.76$	$1.04 \pm 0.41$
<b>LPS (<math>\text{pg}\cdot\text{ml}^{-1}</math>)</b>				
CON	$13.45 \pm 14.77$	$17.30 \pm 18.86$	$9.07 \pm 2.69$	$8.57 \pm 2.84$
PRO	$8.99 \pm 3.77$	$12.47 \pm 3.67$	$7.85 \pm 5.58$	$8.90 \pm 3.79$
PGLn	$9.33 \pm 4.63$	$9.98 \pm 4.44$	$6.54 \pm 3.11$	$11.71 \pm 4.18$
<b>LPS (<math>\text{EU}\cdot\text{ml}^{-1}</math>)</b>				
CON	$0.13 \pm 0.14$	$0.17 \pm 0.18$	$0.09 \pm 0.03$	$0.08 \pm 0.02$
PRO	$0.09 \pm 0.04$	$0.13 \pm 0.04$	$0.07 \pm 0.06$	$0.09 \pm 0.04$
PGLn	$0.09 \pm 0.04$	$0.10 \pm 0.04$	$0.07 \pm 0.03$	$0.09 \pm 0.04$

eHsp72: extracellular heat shock protein 72; LPS: Lipopolysaccharides; PRO: Probiotic; PGLn: Probiotic + Glutamine; CON: Control.

### 3.4 Discussion

#### 3.4.1 Key findings

The key findings from this study were as follows: firstly, PRO and PGLn supplementation had no significant effect on eHsp72 concentration from baseline to pre-race in all conditions ( $p > 0.05$ ), thus hypothesis one must be rejected. In line with hypothesis two, eHsp72 concentration was significantly increased post-race compared to baseline and all other time points (pre-race, 7 d post-race) in all groups ( $p < 0.05$ ); there was no significant effect of group post-race or at any other time point ( $p > 0.05$ ) contrary to hypothesis three. No significant change was seen in LPS concentration from baseline and pre-race to post-race in all groups ( $p > 0.05$ ), and thus hypothesis four is rejected.

#### 3.4.2 eHsp72 response

In contrast to hypothesis one, eHsp72 was not significantly increased from baseline to pre-race in PRO or PGLn. This was unexpected, as previous research has suggested that a key mechanism behind the protective role of PRO (Petrof *et al.*, 2004; Tao *et al.*, 2006) and GLn (Wischmeyer *et al.*, 2001) is the elevated induction of eHsp72. Tao *et al.* (2006) stated that induction of eHsp72 following PRO treatment *in vitro* may be transcriptional in nature, and was shown to induce binding of HSF-1, a key activator responsible for transcription of heat shock genes [section 2.4.1; (Zuhl *et al.*, 2014)]; future related work should include measurement of HSF-1 within their designs to provide mechanistic evidence aligned to this hypothesis. Tao *et al.* (2006) also stated that Hsp genes were the most dramatically upregulated genes in response to PRO treatment, which suggests the induction of eHsp72 to be a key mechanism behind the protective role of PRO supplementation, and stated that PRO exposure of as little as a few minutes was sufficient to initiate signals to the epithelial cells for Hsp induction. Based upon this evidence, the 12 wk supplementation period utilised in the present study far exceeds the 16 h of *in vitro* exposure implemented by Tao *et al.* (2006) and thus was hypothesised to induce a significant increase in eHsp72 concentration, however, this increase did not occur. The PRO and PGLn supplements utilised in this study were commercially available

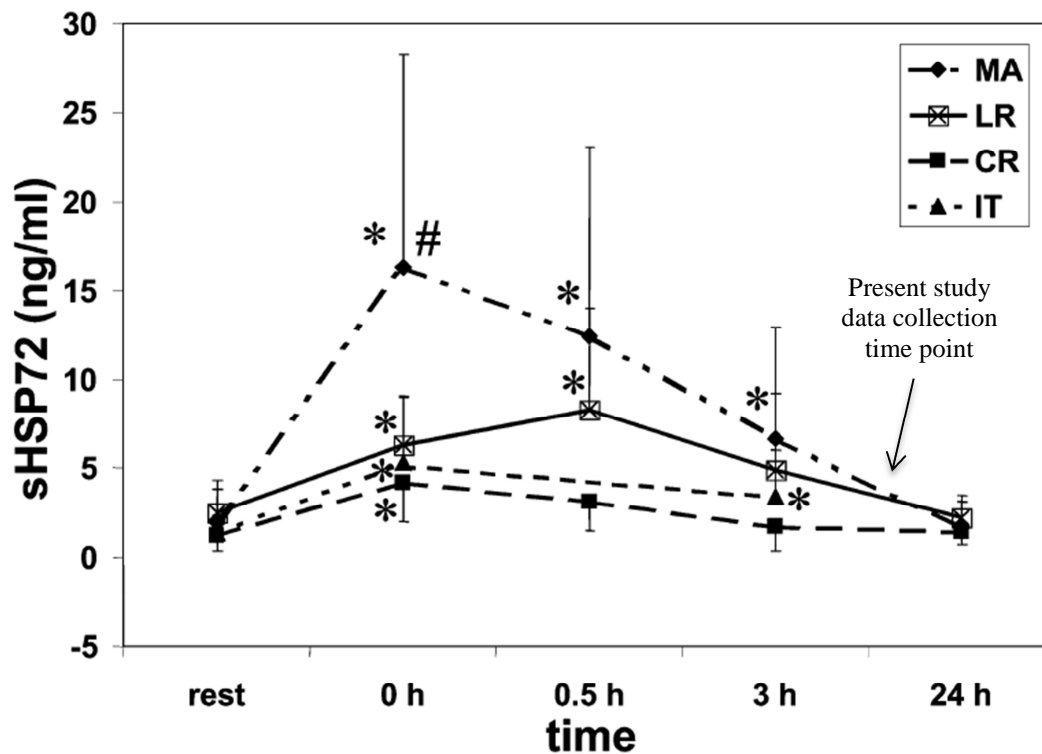
products (see section 3.2.4 and Appendices D and E for details), and thus whilst this aids external validity to current practice, it is possible that additional ingredients included in the supplements could have influenced the outcome of the study (Maughan, 2005). The wide range of available probiotic strains and bacteria, and the lack of quality control of probiotics (Tuomola *et al.*, 2001), make comparisons between probiotic supplementation studies difficult; therefore future research utilising similar experimental designs should develop a standardised and pharmacologically optimised PRO supplement strain (which may likely be hybridised).

In addition, GLn, described as a potent enhancer of the heat shock response [eHsp72 in particular (Wischmeyer, 2002)], has reportedly led to increased HSP70 expression in intestinal epithelial cells, leading to protection against oxidant and heat injury (Wischmeyer *et al.*, 1997). Zuhl *et al.* (2014) demonstrated that 7 d GLn supplementation led to a significant increase ( $p < 0.05$ ) in HSP70 concentration in intestinal cells *in vitro* and, interestingly, prevented exercise-induced GI permeability. Findings from Zuhl *et al.* (2014) also indicated an absence of the HSP70 response to heat stress without GLn, and thus demonstrated i) the key role of this amino acid on HSP70 induction, and ii) the role of HSP70 in cellular protection in intestinal cells. In contrast to previous literature, the present study has displayed unexpected findings; this may be attributable to the prescribed GLn dosage, which was perhaps insufficient to induce a significant eHsp72 response. Previous studies exhibiting an increase in eHsp72 following GLn supplementation have utilised dosages relative to each individual participant, for example Zuhl *et al.* (2014; 2015) implemented a dosage of 0.9 g/kg fat free mass per day for each participant, whereas the present study applied a standardised dose of 5 g/day PGLn for all participants, which contained only 0.9 g of GLn per dose. This therefore may have been an insufficient dosage to induce an eHsp72 response in the PGLn group similar to that seen in previous studies (Zuhl *et al.*, 2014; 2015).

Although no significant change in eHsp72 concentration was found from baseline to pre-race, a significant elevation in eHsp72 concentration was shown post-race. This is in accordance with previous literature (Gibson *et al.*, 2014; Gomez-Merino *et al.*, 2006; Lancaster *et al.*, 2004; Suzuki *et al.*, 2006; Walsh *et al.*, 2001), which has



demonstrated the significant impact of exercise, heat stress, and the combination of both stressors upon the heat shock response. These findings demonstrate that participation in the MDS imposed a substantial level of stress upon all participants, subsequently inducing an overall mean increase in eHsp72 concentration of 124% from baseline to post-race. Due to the novelty of data collected in the present study, direct comparisons to previous research are difficult, and to the author's knowledge, only two previous studies have reported the eHsp72 response to ultra-endurance exercise performance (Gomez-Merino *et al.*, 2006; Suzuki *et al.*, 2006). These studies demonstrated an increased eHsp72 concentration of ~ 2200% (Suzuki *et al.*, 2006) and ~ 1674% (Gomez-Merino *et al.*, 2006) following an ironman triathlon and 100 km run, respectively. Evidently, the post-race increase of 124% from baseline in the present study was less substantial than those ascertained in these previous studies, however, it is clear to see that a significant level of stress has been imposed upon the participants, to trigger the observed increase in eHsp72 concentration. One possible explanation for this seemingly less substantial increase in post-race eHsp72 concentration in the present study is the timing of post-race blood sample collection. Due to the logistical issues of conducting field based research at the MDS, post-race samples were collected 6 - 8 h post-race, whereas previous research has collected samples immediately upon exercise completion (Gomez-Merino *et al.*, 2006; Suzuki *et al.*, 2006). As shown in Figure 3.8, Fehrenbach *et al.* (2005) previously demonstrated a significant increase in post-exercise eHsp72 concentration *in vivo*, which was shown to decrease back to baseline levels within 24 h post-exercise. As indicated in Figure 3.8, data in the present study was likely collected during the return to baseline concentration phase, and therefore may not provide an accurate representation of the eHsp72 response to ultramarathon exercise in extreme heat. The findings of Fehrenbach *et al.* (2005) indicate the time-course of changes in eHsp72 concentration following exercise, which may aid understanding regarding the less substantial increase observed in the present study when compared to previous research (Gomez-Merino *et al.*, 2006; Suzuki *et al.*, 2006). It can therefore be postulated that if samples were collected immediately post-race in the present experimental design, a greater eHsp72 concentration in comparison to those collected at 6 - 8 h post-race may have been seen.



**Figure 3.8.** Changes of soluble HSP72 (at rest, 0 h, 0.5 h, 3 h, and 24 h after exercise) in the plasma of endurance athletes after exercise of different intensity and duration. MA= marathon, LR = long run, CR = continuous run, IT = interval training were compared. \*Denotes significant differences between pre- and post-exercise data,  $p < 0.05$  (Fehrenbach *et al.*, 2005).

In addition, it is possible that a habituation effect may have occurred due to the multi-day nature of the event. Following 7 consecutive days of exercise heat-stress in extreme environmental conditions, participants would likely begin to acclimate to the extreme conditions. Full heat acclimation has been indicated to require 5-10 days (Sawka *et al.*, 2011), thus by the 7<sup>th</sup> consecutive day of exercise, it is likely that participants would have developed a level of acclimation to these conditions. Consequently, resting eHsp72 concentration prior to the commencement of the final MDS stage would likely have been greater than recorded pre-race values (McClung *et al.*, 2008). This speculation could aid understanding regarding the less substantial increase in eHsp72 post-exercise in comparison to previous ultramarathon research (Gomez-Merino *et al.*, 2006; Suzuki *et al.*, 2006). Boshoff *et al.* (2000), Gjøvaag and Dahl (2006), and Sandström *et al.* (2008) have shown that participants with high pre-exercise Hsp70 concentration displayed smaller increases in post-exercise values *in vivo*. Sandström *et al.* (2008) also demonstrated an increase in basal Hsp70 over a 15-day acclimation period, which indicates the possibility of elevated resting eHsp72

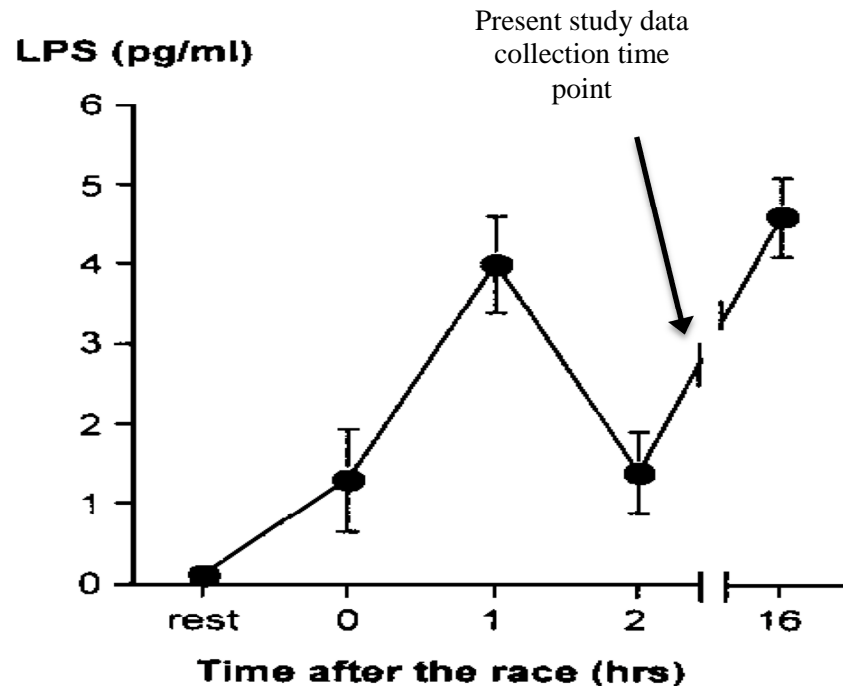
prior to commencement of the final stage in the present study. Although evidently not directly quantifiable in this study, it can be speculated that prior to commencement of the final race stage, resting eHsp72 would have been greater than the recorded pre-race values, and thus post-race eHsp72 may have displayed a less substantial increase than those seen in previous studies (Gomez-Merino *et al.*, 2006; Suzuki *et al.*, 2006) due to the potential role of heat acclimation (Sandström *et al.*, 2008). Evidently, the inclusion of daily data collection upon stage completion would provide the data required to quantify this speculation, which should be considered for future studies investigating multi-day ultra-endurance events.

To summarise PRO and PGLn supplementation regimes were ineffective to i) increase basal eHsp72 values pre-race and ii) ameliorate the eHsp72 response seen in all groups post-race.

### 3.4.3 LPS Response

No significant change ( $p > 0.05$ ) in circulatory LPS concentration from baseline to post-race was observed within the present study. This is in contrast to hypothesis four and opposes the findings from a number of previous studies in which endurance (Ng *et al.*, 2008) and ultra-endurance (Costa *et al.*, 2014; Jeukendrup *et al.*, 2000) exercise have initiated a significant increase in circulatory LPS concentration. Jeukendrup *et al.* (2000) demonstrated a significant rise in LPS concentration and mild endotoxemia in 68% of athletes following an ironman distance triathlon. However, whilst Jeukendrup *et al.* (2000) demonstrated that endotoxemia was present in 68% of athletes 1 h post-race, this reduced to just 19% 2 h post-race, before increasing further to 79% 16 h post race. Figure 3.9, adapted from Jeukendrup *et al.* (2000), demonstrates changes in post-race LPS concentration, and indicates the time-point at which data was collected in the present study. As shown, LPS concentration at this time-point was clearly lower than the LPS concentration observed at 1h post-race. This suggests that samples collected between 6 – 8 h post-race in the present study may have occurred during the trough phase, and thus data collection immediately/ within 1 h post-race could have demonstrated higher LPS values with the present study. However, Jeukendrup *et al.* (2000) did not collect hourly samples, and to the author's knowledge no study has been conducted to

validate the time-course of LPS translocation following exercise, and thus this theory is only speculative. Future research exploring the time-course of post-exercise LPS translocation should utilise the standardised and pharmacologically optimised PRO supplement proposed earlier within this section.



**Figure 3.9.** LPS concentration before, and at several time points following, an ironman triathlon, demonstrating the potential LPS response at the time of data collection in the present study; adapted from Jeukendrup *et al.* (2000).

In addition to this, it may be postulated that the beneficial effects of the supplementation regimes were beginning to reduce towards the end of the race. Previous studies have established that the typical time-course for passage of probiotic bacteria through the GI tract is 3-8 days (Klijn *et al.*, 1995), and it is still unclear as to whether these remain metabolically active in the GI tract, making it difficult to conclude whether the effects of the supplementation were present during the MDS. It is possible that the effect was still present, previous research investigating the effects of probiotic supplementation utilising repeated measures designs have commonly implemented washout periods of between 3 (Shing *et al.*, 2014) and 6 (Rosenfeldt *et al.*, 2004) weeks, which may suggest that the effects remain for more than 7d following the termination of supplementation. However, to

the author's knowledge, there is a lack of evidence to demonstrate the duration that the effect persists, thus this is purely speculation, and further research is required to obtain a greater understanding.

Furthermore, although endotoxemia is reportedly common amongst endurance and ultra-endurance athletes (Ter Steege *et al.*, 2008), it has been shown that “training” the gut is a potential method by which levels of GI discomfort and LPS translocation/endotoxemia can be significantly reduced and/or avoided during these events (Carrio *et al.*, 1989; Selkirk *et al.*, 2008). Murray (2006) stated that proper training and nutrition reduces the risk of GI discomfort during exercise by maintaining adequate perfusion of the splanchnic vasculature – a key contributor to intestinal wall damage and subsequent LPS translocation [section 2.3.2; Moses (2005)]. Carrio *et al.* (1989) reported enhanced gastric emptying rates in endurance athletes, with support from Gisolfi (2000), stating that splanchnic blood flow is greater in trained athletes at any given workload, and thus improved gut barrier function commonly occurs with training as a result of enhanced blood flow. Selkirk *et al.* (2008) also demonstrated significantly lower levels of plasma LPS in trained individuals compared to untrained at a given absolute  $T_{core}$  during exercise in the heat. This training effect may be further enhanced in events, such as the MDS, where exercise is performed on consecutive days, again highlighting the possibility of the aforementioned habituation effect, and the requirement of daily sample collection in future research studies to quantify this postulation. These studies suggest that trained individuals are less likely to suffer from endotoxemia during exercise heat-stress.

The participants in this study were well trained (overall average pre-race  $\dot{V}O_{2max}$   $57.55 \pm 2.09$  ml.kg.min<sup>-1</sup>), and thus it could be suggested that the lack of increase in post-race circulatory LPS concentration may be attributed to a sufficient level of splanchnic blood flow during exercise, as a result of enhanced training status, which may subsequently reduce the severity of GI damage. A significant increase ( $p < 0.05$ ) in  $\dot{V}O_{2max}$  was displayed from baseline to pre-race in all groups, indicating a significant training effect over time. Additionally, as no difference in GI discomfort between groups was demonstrated throughout the MDS, it is likely that the training status of the individuals was a more potent contributor to the GI response, as no

effect of group ( $p > 0.05$ ) was observed for overall fitness based upon average  $\dot{V}O_{2\max}$ . It is important to note that gut blood flow was not measured in this study and therefore further research into this theory should be conducted, potentially utilising measures of splanchnic blood flow, in addition to daily sample collection, to demonstrate the effect of ultra-endurance exercise on the trained athlete's gut.

As no significant increase in LPS concentration ( $p > 0.05$ ) occurred in the present study, and no significant correlation ( $r^2 = -0.122$ ,  $p > 0.05$ ) was found between eHsp72 and LPS concentration following exercise, it is difficult to draw conclusions regarding the relationship between the two variables. These findings suggest no significant interaction was present between LPS and eHsp72 concentration. Previous research has highlighted the role of eHsp72 on LPS tolerance [section 2.3.3 (Aneja *et al.*, 2006)], higher concentrations of eHsp72 have been associated with increased LPS tolerance through reduced pro-inflammatory cytokine release in response to LPS (Ferat-Osorio *et al.*, 2014). Thus, it can be speculated that the elevated eHsp72 concentration observed post-race may have contributed to an elevated LPS tolerance. This cannot be quantified in the present study due to the absence of pro-inflammatory cytokine analysis (i.e. TNF- $\alpha$ , IL-6), and thus this postulation also requires further investigation.

In addition, despite no significant correlation between eHsp72 ( $r = -0.008$ ,  $p = 0.962$ ) or LPS ( $r = 0.128$ ,  $p = 0.470$ ) concentration and MDS finishing time, a significant effect of group on finishing time was displayed, whereby the PRO group completed the race significantly faster than CON. As stated, two participants were disqualified from the race due to the failure to complete a stage within the required time, however, when analysed both including and excluding these participants, the CON group remained significantly slower on average in comparison to the PRO group, suggesting that the disqualified participants' time did not alter the results of the analysis. These findings are difficult to explain, as there was no effect of group on post-race eHsp72 or LPS concentration and thus these variables cannot be confirmed as the cause of the observed difference in finishing times. Furthermore, no significant difference in fitness levels was demonstrated between groups, which therefore suggests that a confounding variable, potentially related to the ingredients

of the PRO supplementation (as postulated in section 3.4.2), was responsible for this outcome. Due to the prolonged duration of the intervention, participants were not blinded to condition, which may have caused a demotivational response, and potentially led to impaired performance in the CON group through a reverse placebo, also known as nocebo, effect (Carlino *et al.*, 2014). However, it is difficult to reach a clear conclusion regarding the role of supplementation upon performance, as this was not a main outcome variable of the present study, additional research is therefore required to further investigate the effect of this supplementation upon ultramarathon performance, in order to demonstrate whether supplementation for GI distress can have a significant impact upon overall performance.

In addition to this, the baseline LPS concentrations (Table 3.2) were higher than expected in all groups. As endotoxemia is defined as an LPS concentration  $> 5\text{pg/ml}$ , the results of the present study indicate that all participants were in a state of endotoxemia at baseline. This result was unexpected and may indicate the possibility of LPS sample contamination in this instance. However, all procedures during testing followed the correct guidelines, in an attempt to ensure that all samples were protected against contamination, and as a result, it was deemed appropriate to include these results as baseline levels in the present study, as contamination is only one possible explanation as to the cause of these unexpected results. Although it is recognised that these results are seemingly high in comparison to previous research, the author deemed it appropriate to report the data, and display results as a percentage change from these baseline values, as all procedures and protocols throughout the study were followed correctly. Additionally, whilst the majority of previous literature would suggest that these results are high, this is not the first study to display above average baseline values. Lim *et al.* (2009) displayed pre-exercise values  $>10\text{pg.ml}^{-1}$ , which supports the data in the present study. Future research should aim to investigate a clear explanation as to why baseline LPS values may be high in certain individuals. Future research should also focus upon minimising the risk of contamination, by ensuring that endotoxin-free tubes are used and are kept in a sterile environment, gloves are worn at all times when handling samples, and the appropriate pipette tips are used during analysis.

#### 3.4.4 Synthesis of findings

The findings observed in the present study primarily demonstrate the key role of ultra-endurance exercise on the eHsp72 response, and suggest that PRO and PGLn supplementation may be ineffective nutritional interventions to induce an increase in basal eHsp72 concentration in humans. Additionally, previous research is largely in agreement that participation in ultra-endurance events stimulates a substantial increase in circulatory LPS concentration in single day events (Brock-Utne *et al.*, 1988; Jeukendrup *et al.*, 2000). The severe level of stress imposed upon the gut during such exercise leads to intestinal epithelial cell and tight junction damage (Van Wijck *et al.*, 2011), causing a subsequent increase in GI mucosal membrane permeability, and thus an undesirable increase in LPS translocation (Berkes *et al.*, 2003). However, the multi-day ultra-endurance exercise undertaken in the present study did not lead to a significant increase in LPS concentration across all groups, which was an unexpected outcome. This may be attributable to a habituation and acclimation effect across the duration of the event, which should be measured in future studies via daily sample collection. The following sections will explain the practical applications (section 3.4.5) of the present study, and will subsequently discuss the experimental limitations (section 3.4.6) and highlight recommendations for future research (section 3.4.6) based upon these findings. The novel data collected in the present study should be utilised to enhance future research design and develop the currently limited knowledge surrounding this field of research, with a particular focus on ultra-endurance exercise.

#### 3.4.5 Application of findings

This study aimed to investigate the effects of chronic probiotic supplementation on LPS translocation and the eHsp72 response following an ultra-endurance event in extreme environmental conditions. As stated in section 2.2.1, ultra-endurance events are becoming increasingly popular (Knechtle *et al.*, 2011), and, as a result, investigations into methods that could attenuate detrimental side effects and ultimately optimise performance are highly desired. The findings from the present study are in contrast to previous research, which has inferred a significant beneficial



role of probiotic supplementation upon GI permeability and athletic performance (Kekkonen *et al.*, 2007; Lamprecht *et al.*, 2012; Shing *et al.*, 2014). The present study indicates that chronic PRO and PGLn supplementation interventions are ineffective in relation to the LPS and eHsp72 response to multi-day ultra-endurance events. It is suggested that an adequate level of training (likely heat acclimation) prior to the event could be sufficient to blunt the detrimental LPS response commonly associated with ultra-endurance events, and subsequently prevent the previously established GI-related decrement in performance (Riddoch and Trinick, 1988).

#### 3.4.6 Experimental limitations and future research

Limitations have been highlighted in relation to the experimental procedures of the present study, which should be considered when interpreting results and undertaking future research. Due to the collection of novel data with extremely high external validity in a field-based research design, control of certain aspects of the study were undesirably compromised in places. The principal limitations of the present study were the time period elapsed between race completion and venous blood sample collection, and the frequency of sample acquisition within race. Ideally, daily venous blood sample collection would have occurred immediately prior to and post stage completion (or within ~30 min) in accordance with previous research (Gomez-Merino *et al.*, 2006; Suzuki *et al.*, 2006) to reduce the influence of confounding factors (as discussed in sections 3.4.2 and 3.4.3) on plasma samples and to increase understanding regarding the changes in eHsp72 and LPS concentration throughout the duration of the event. However, the logistical issues associated with field-based research heavily influenced post-race blood sample collection in the present study. As a result, post-race blood samples, originally scheduled for ~ 1-2 h post-exercise, were delayed to a range of 6 – 8 h post-exercise, which may have resulted in alterations in the observed LPS and eHsp72 concentration. Future research should aim to eliminate this confounding factor, by collecting post-race samples as close to race completion as possible (preferably within 1 h post exercise). This will give a truer representation of the eHsp72 and LPS response to the exercise undertaken. This limitation highlights a major confounding factor in the present study and provides a

potential explanation for the observed, seemingly low, post-race LPS concentration in all groups.

Furthermore, the analysis of variables at additional time points could contribute to obtaining more detailed, reliable results regarding the response to multi-day ultramarathon performance. Future research should investigate the eHsp72 and LPS response to each individual stage, as well as to the overall race, by collecting daily samples upon stage completion. This would provide further novel data, demonstrating the impact of consecutive bouts of prolonged endurance running in extreme environmental conditions on GI permeability, and the eHsp72 response. Due to the logistical restraints of the MDS (taking place in the Sahara Desert - a restrictive environment for data collection), collection of daily samples and immediate post-race samples was unfortunately not possible. Future studies investigating multi-day ultra-endurance events in more accessible environments should consider this recommendation, in order to obtain more robust, detailed information regarding these specific cellular responses. In addition to this, it may also be suggested that future research should consider implementing sample collection through alternative methods, such as urine or saliva samples. However, whilst it is possible that these samples could be collected without experimenter supervision, it is likely that accuracy of samples would be affected by lack of control and the impact of sterility upon samples. Future research should investigate a method by which within-race samples can be collected, whilst reducing the risk of impaired data output. Additional markers, such as iHsp72 and pro-inflammatory cytokine analysis, could also be included in future studies; these markers could provide further understanding regarding the immunological and protective role of the heat shock response following such exercise, with greater focus upon the link between eHsp72 and innate immunity (Asea, 2003).

The PRO and PGLn supplements and dosage implemented in the present study may have also strongly influenced the outcome. As stated, it is possible that these commercially available products contained additional ingredients that may have acted as confounding factors (Maughan, 2005), and subsequently influenced the observed eHsp72 and LPS response. This limitation should be addressed in future research, whereby the development of a standardised and pharmacologically

optimised PRO supplement strain is essential in order to improve reliability and generalisability. This could subsequently improve the quality control of PRO supplementation, an issue previously highlighted by Tuomola *et al.* (2001), and could therefore reduce the impact of confounding variables upon results.

Moreover, the implemented PRO and PGLn dosage may have been insufficient to induce the hypothesised eHsp72 and LPS response. The use of a standardised dose for all participants in the present study may have caused varying responses between individuals, whereas previous *in vivo* research has commonly implemented a dose relative to participants' body mass (Zuhl *et al.*, 2014; 2015) which may have led to a more specific and generalisable response between participants. Future research should consider investigating the optimal dose required to initiate the proposed eHsp72 response, which could consequently be implemented in future research designs, in order to enhance generalisability across the field.

It is also possible that as participants were not blinded to group assignment, a placebo effect (Carlino *et al.*, 2014) may have taken place in the CON group, and may have influenced overall race performance, however, this is purely speculation. Future research should consider the effect of blinded groups upon findings, in combination with the implementation of a placebo supplement in the CON group, in order to reduce the risk of a placebo effect taking place.

As aforementioned (section 2.3.3), research has demonstrated a key role of eHsp72 on LPS tolerance (Aneja *et al.*, 2006). A greater insight into the *in vivo* relationship between eHsp72 and LPS could be elucidated via the inclusion of pro-inflammatory cytokine analysis (i.e. TNF- $\alpha$ , IL-6), to infer a greater understanding regarding the role of eHsp72 upon LPS tolerance in humans. Participation in a highly stressful, multi-day event, such as the MDS, may induce LPS tolerance in humans; however, this cannot be quantified in the present study, due to the absence of pro-inflammatory cytokine analysis. This highlights the need for future research to build upon the present study, and to enhance the findings to broaden understanding regarding the relationship between the eHsp72 and LPS response to ultra-endurance exercise.

In addition, Lee *et al.* (2015) recently suggested that the commercially available eHsp72 ELISA kit (HSP70 high sensitivity ELISA kit, Enzo Life Sciences, Exeter, UK) utilised in the present study offers less precise results in comparison to a newly available alternative ELISA kit (ENZ-KIT-101-001 HSP70 Amp'd® ELISA). Lee *et al.* (2015) subsequently recommended that utilizing the new ENZ-KIT-101-001 HSP70 Amp'd® ELISA kit will provide a more accurate measure of resting eHsp70 quantification, due to the increased sensitivity of this assay. Thus, it is suggested that in line with these recommendations, future research should utilize this newly developed ELISA kit. Use of this kit could offer more accurate quantification of eHsp72 concentration, which, in the present study, could have provided a clearer insight regarding the effect of PRO and PGLn supplementation on the eHsp72 response pre-race.

Finally, greater control over the training and nutritional programmes undertaken by participants over the 12 wk intervention period would have allowed for enhanced control and reliability regarding the source of specific conclusions of the investigation. Future studies should consider implementing a standardised training and nutrition programme for all experimental groups, in order to reduce the impact of confounding variables. In addition, the inclusion of these programmes would elucidate a clearer understanding regarding the role of nutrition and training upon LPS translocation and the subsequent eHsp72 response to ultra-endurance exercise.

### **3.5 Conclusion**

In conclusion, the present study has demonstrated that nutritional interventions of chronic PRO and PGLn supplementation are not required in order to reduce LPS translocation and GI discomfort during ultra-endurance performance in extreme heat. A high level of fitness may induce physiological adaptations that could improve overall gut health, and the increased eHsp72 concentration in response to exercise may offer cytoprotection and aid the reduction of GI discomfort during performance. Future investigations should aim to build upon this field-based research, but should include additional markers of GI damage, and pro-inflammatory cytokine analysis (i.e. TNF- $\alpha$ , IL-6), to offer a greater level of understanding regarding the relationship between eHsp72 and LPS concentration. Future research should also aim to collect

samples immediately/ within 1 h of race completion in order to obtain a more accurate representation of the physiological and biological responses to ultra-endurance exercise in extreme environments.

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## CHAPTER 5: Appendices

### Appendix A

#### Participant Information Sheet

#### Section A: The Research Project

**Title of project:** Chronic Probiotic Supplementation and its Effects on eHsp72 and LPS concentration following a Desert-based Ultramarathon

1. **Purpose and value of study:** The purpose of the study is to investigate the impact of nutritional intake prior to a multi-day ultra-endurance event. With the ever-increasing numbers of athletes of all levels competing in ultra-endurance events a greater understanding of the demands placed on the body by these events is required.
2. **Invitation to participate:** You are being invited to take part in a research study. Before you decide whether to do so, it is important that you understand the research that is being done and what your involvement will include. Please take the time to read the following information carefully and discuss it with others if you wish. Do not hesitate to ask us anything that is not clear or for any further information you would like to help you would like to help make your decision. Please do take your time to decide whether or not you wish to take part. Thank you for reading this.
3. **Who is organising the research:** The research is being organised by Craig Suckling under the supervision of Dr Justin Roberts and Dr Dan Gordon from Anglia Ruskin University in collaboration with Hannah Marshall and Dr Lee Taylor from the University of Bedfordshire.
4. **What will happen to the results of the study:** If you agree to take part, your results will be stored on a password protected computer and portable disk drive, with your name and other details removed. Blood samples will be collected, stored securely and disposed of under Human Tissue Authority regulations. The samples will be analysed for endotoxin units and heat shock proteins only. The research team at Anglia Ruskin University will keep all paper data securely. We expect the data will be published in a scientific journal and presented at a scientific conference. On publication of the results it will not be possible to identify individual participants.
5. **Source of funding for the research:** The research is funded by the lead researcher.
6. **Contact for further information:** [craig.suckling@student.anglia.ac.uk](mailto:craig.suckling@student.anglia.ac.uk) or [Hannah.marshall1@study.beds.ac.uk](mailto:Hannah.marshall1@study.beds.ac.uk)

#### Section B: Your Participation in the Research Project

1. **Why you have been invited to take part:** You have been invited to participate in the research because you have entered the 2015 Marathon des Sables and expressed an interest in taking part in the research project.

2. **Whether you can refuse to take part:** You are under no obligation to participate in the study.
3. **Whether you can withdraw at any time, and how:** You are free to withdraw without reason and without penalty from the study at any time. If you wish to withdraw from the study please complete the withdrawal form on the bottom of the consent form and return it to the research organiser.
4. **What will happen if you agree to take part (brief description of procedures/tests):**

The study will take place in 3 phases:

**PRE** event testing will take place at Anglia Ruskin University (January 8<sup>th</sup> - 13<sup>th</sup> and March 26<sup>th</sup> - 31<sup>st</sup>) Testing slots can be booked on a first come first served basis through the lead researcher.

**EVENT** testing will take place during the 2015 Marathon Des Sables (3<sup>rd</sup> - 13<sup>th</sup> April) at Berbere Hotel, Ouarzazate. As you return upon completion of the race.

**POST** event testing will take place at Middlesex University (16<sup>th</sup> – 20<sup>th</sup> April). Testing slots can be booked on a first come first served basis through the lead researcher.

If you decide to take part in the study you will be required to attend all 3 phases; details of the procedures you will undergo are detailed below.

Body composition assessment (All phases) during which your height (m), Body mass (kg) will be measured. You will also undergo body fat estimation using skinfold and segmental measures. Please note that you will be required to wear loose fitting sports kit (female participants a sports bra is required); the procedure can be performed by a gender-matched researcher if requested. (Approximate time commitment 20-40 mins)

Resting blood sample (All phases) a single blood sample (3x4ml samples) will be collected from your arm. The procedure will be carried out by a trained phlebotomist. You will be required to refrain from food/drink (except water) for 6 hours prior to the test. (Approximate time commitment 10 mins)

Maximal oxygen uptake test (VO<sub>2MAX</sub>) (Pre phase only) a short duration incremental exercise treadmill test for the determination of the highest absolute oxygen uptake value. You will run on a treadmill in 2 minute stages of progressive intensity, while oxygen uptake is measured. (Approximate time commitment 20-40 mins)

Nutritional Intervention you will be randomly assigned to 1 of 3 experimental groups for the duration of the study. Depending on the group

you are assigned to you may be required to take a supplement or maintain habitual diet. Each group will be briefed individually as to the exact requirements (approximate time commitment none)

5. **Whether there are any risks involved (e.g. side effects from taking part) and if so what will be done to ensure your wellbeing/safety:** Research of this nature has a number of potential risks, however every effort has been made to minimise the nature of these risks and ensure your wellbeing.

Body composition - possible but highly unlikely risks include: discomfort or skin irritation from skinfold/ segmental location and assessment. All research testers are experienced in skinfold and anthropometrical assessment.

Resting blood collection – possible risks include: dizziness/fainting/ nausea from blood collection procedure; localised bruising. All venepuncture procedures will be undertaken by qualified phlebotomists only.

Maximal exercise testing – potential risks may include: fatigue/ tiredness and localised muscle soreness following exercise exertion. Participants will complete appropriate warm up and cool down, two testers who are first aid trained will be conducting the test.

Nutritional supplementation (12 weeks) – possible acute side effects (nausea, dizziness, bloating, gastrointestinal distress from consumption). All nutritional products will be supplied by a highly reputable clinical nutrition company: Biocare Nutrition Ltd., and are commercially available to the UK general public.

Risk assessments have been carried out and reviewed at each testing location. Throughout the testing procedures standard operating procedures for each location will be followed by the research team.

6. **Agreement to participate in this research should not compromise your legal rights should something go wrong:** Participation does not compromise your legal rights should anything go wrong. Suitability of the entrants to participate in the event and full liability falls under the race organiser's regulations and is not part of the study.
7. **Whether there are any special precautions you must take before, during or after taking part in the study:** All participants are recommended to avoid strenuous exercise 48 hours prior to PRE event testing session. Avoid food and drink (except water) for 6 hours prior to PRE and POST event resting blood samples and for 2 hours prior to EVENT resting blood sample. No use of nutritional supplements that promote/improve gut health directly or indirectly for the duration of the study, the lead researcher will provide details of supplements that cannot be used.
8. **What will happen to any information/data/samples that are collected from you:** Any information/data/samples collected from you as part of the study will be stored anonymously in accordance with current legislation and

Anglia Ruskin University policy. The blood samples will be analysed to assess the levels of endotoxin units and heat shock proteins present. The study will be written up using the information and data collected as part of a PhD/Masters thesis and for publication in a scientific journal. It will not be possible to identify individual participants from the published work.

- 9. Whether there are any benefits from taking part:** By taking part in the study you will receive the following benefits:

High quality fitness assessment including maximal oxygen uptake ( $VO_{2MAX}$ )  
Generic dietary advice.

Free accurate assessment of your body composition.

Access to two Marathon Des Sables finishers

- 10. How your participation in the project will be kept confidential:** All subjects will be randomly assigned a numeric code; all data will be collected and stored under this code. Collected data will be stored in a secure manner to maintain confidentiality and anonymity.



## Appendix B

### Consent Form

NAME OF PARTICIPANT:

**Title of the project:** Chronic Probiotic Supplementation and its Effects on eHsp72 and LPS concentration following a Desert-based Ultramarathon

Main investigator and contact details: Craig Suckling [Craig.suckling@student.anglia.ac.uk](mailto:Craig.suckling@student.anglia.ac.uk) ,  
Hannah Marshall: [Hannah.marshall1@study.beds.ac.uk](mailto:Hannah.marshall1@study.beds.ac.uk)

Members of the research team: Dr Justin Roberts, Dr Dan Gordon, Dr Mike Roberts, Dr Lee Taylor.

1. I agree to take part in the above research. I have read the Participant Information Sheet for the study. I understand what my role will be in this research, and all my questions have been answered to my satisfaction.
2. I understand that I am free to withdraw from the research at any time, for any reason and without prejudice.
3. I have been informed that the confidentiality of the information I provide will be safeguarded.
4. I am free to ask any questions at any time before and during the study.
5. I have been provided with a copy of this form and the Participant Information Sheet.

Data Protection: I agree to the University processing personal data which I have supplied. I agree to the processing of such data for any purposes connected with the

Research Project as outlined to me\*

Name of participant (print).....  
Signed.....  
Date

If you wish to withdraw from the research, please complete the form below and return to the main investigator named above.

I WISH TO WITHDRAW FROM THIS STUDY: Signed:

\_\_\_\_\_ Date: \_\_\_\_\_

## Appendix C

### Participant Health Screen Questionnaire

#### PRE-PARTICIPATION HEALTH SCREEN QUESTIONNAIRE

##### Name of participant:

It is important when having volunteered as a participant for this study, and having read the briefing sheet for all participants that you answer the following questions. Please do not answer any questions if you consider them intrusive, instead speak to Dr J Roberts.

- 1) Are you currently aged between 18-60 years, and consider yourself recreationally active?

Yes	No
-----	----

- 2) Are you pregnant?

Yes	No
-----	----

- 3) Do you suffer from diabetes or low blood sugar (hypoglycaemia)?

Yes	No
-----	----

- 4) Is there any history of diabetes or low blood sugar in your immediate family?

Yes	No
-----	----

- 5) Do you suffer from high blood pressure, or any heart problems?

Yes	No
-----	----

- 6) Do you often get dizzy, or do you know that you have low blood pressure?

Yes	No
-----	----

- 7) Is there any history of heart disorders or heart disease in your immediate family?

Yes	No
-----	----

- 8) Do you, or have you suffered from any blood related disorders, or have you any issues related to blood taking?

Yes	No
-----	----

- 9) Have you suffered from any recent viral infections?

Yes	No
-----	----

- 10) Are you suffering from any musculo-skeletal injury?

Yes	No
-----	----

- 11) Are you currently taking any medication (over the counter, or prescription)?

Yes	No
-----	----

(You do not need to answer “Yes” if you are an asthmatic with an inhaler available; or if taking a contraceptive pill)

- 12) Have you ever been told that you should not exercise?

Yes	No
-----	----

- 13) Do you feel fully fit, and eager to act as study participant?

Yes	No
-----	----

- 14) Are you under the influence of alcohol or any other psycho-active substance?

Yes	No
-----	----

Is there any reason, not stated above, why you cannot take part as a participant in this project?

Yes	No
-----	----

Signature.....

Date:

Checked by (Name):

Date:

## Appendix D

COMMERCIALLY SENSITIVE INFORMATION STRICTLY FOR INTERNAL USE ONLY

### PRODUCT MASTER FILE

#### FULL PRODUCT SPECIFICATION

<b>PRODUCT NAME</b>	Bio-Acidophilus Forte
<b>PRODUCT TYPE</b>	Vegetable Capsules
<b>CATEGORY</b>	Probiotics
<b>ONLINE/ PRICE LIST DESCRIPTOR</b>	BioAcidophilus Forte (Probiotic) 30 Caps BioAcidophilus Forte (Probiotic) 60 Caps

PRODUCT DESCRIPTOR (on label under the product name)

Professional Potency Friendly Bacteria
--

PRODUCT INFORMATION PER DAILY INTAKE (1Capsule)

#### Ingredient Amount

*Lactobacillus acidophilus* 100mg  
(CUL-60 & CUL-21)  
Fructooligosaccharides (F.O.S.) 60mg  
*Bifidobacterium bifidum* (CUL-20) 22.2mg  
& *Bifidobacterium lactis* (CUL-34)  
Providing a total of 30 billion viable proprietary organisms

#### INGREDIENTS

*Lactobacillus acidophilus*, *Bulking Agent (Cellulose)*, *Capsule Shell (Hydroxypropyl Methylcellulose)*, *Fructooligosaccharides*, *Bifidobacterium bifidum* & *Bifidobacterium lactis*, *Anti-Caking Agents (Silicon Dioxide and Magnesium Stearate)*.

FULL ALLERGEN DETAIL:

Added Yeast	no	GM Free	yes	Dairy Free	yes	Low Salt	-
Wheat Free	yes	With Added Sugar	yes	With Added Salt	no	No Salt	-
Gluten Free	yes	No Added Sugar	-	Kosher	no	Halal	no

SUITABILITY:

Vegetarian	yes	Vegan	yes	Organic	-	Can Open capsule	yes
Pregnancy	yes	Breastfeeding	yes	Children	-		

LABEL CAUTIONS/ADVISORY STATEMENTS:

If you are under medical supervision, please consult a doctor before use

This product should not be used as a substitute for a varied and balanced diet and healthy lifestyle.

Do not exceed the stated recommended daily intake.

Do not purchase if the seal is broken.

Keep out of reach of children.

Refrigerate below 4oC and avoid direct sunlight and heat.

## Appendix E

COMMERCIAL SENSITIVE INFORMATION STRICTLY FOR INTERNAL USE ONLY

### PRODUCT MASTER FILE

#### FULL PRODUCT SPECIFICATION

<b>PRODUCT NAME</b>	GI Complex
<b>PRODUCT TYPE</b>	Powder
<b>CATEGORY</b>	Gastrointestinal
<b>ONLINE/ PRICE LIST DESCRIPTOR</b>	GI Complex 165g

PRODUCT DESCRIPTOR (on label under the product name)

L-Glutamine, N.A.G and Probiotic Complex

PRODUCT INFORMATION PER DAILY INTAKE per 5g dose (2 teaspoons)

Ingredient	Amount	% EC	RDA*
Vitamin A 5000iu	1500µg	RE <sup>†</sup>	188
Vitamin E 45iu	30mg	α-TE <sup>††</sup>	250
Vitamin C	180mg		225
Magnesium	12.3mg		3.3
Zinc	20mg		200
Whey Protein	1800mg	-	
L-Glutamine	900mg	-	
Fructooligosaccharides (FOS)		500mg	-
N-Acetyl Glucosamine	435mg	-	
Ginger Powder	300mg	-	
Evening Primrose Oil	33mg	-	
(Providing 3.3mg Gamma Linolenic Acid)			
<i>Lactobacillus acidophilus</i> (CUL-60)	40.5mg	-	
CUL-60 & CUL 21)			
<i>Lactobacillus salivarius</i> (CUL-61)	25mg	-	
<i>Bifidobacterium bifidum</i>	3.5mg	-	
(CUL-20) <i>Bifidobacterium lactis</i> (CUL-34)			
(providing a total of 10 billion viable organisms)			

RE<sup>†</sup> = Retinol Equivalent

α-TE<sup>††</sup> = Alpha Tocopherol Equivalent

RDA = Recommended Daily Allowance

#### INGREDIENTS

Whey Protein Concentrate<sup>1</sup>, L-Glutamine, Fructooligosaccharides (FOS), N-Acetyl Glucosamine<sup>2</sup>, Ginger Powder (*Zingiber Officinale* root), Vitamin C (as Magnesium Ascorbate), Zinc Citrate, Vanilla Flavour, *Lactobacillus acidophilus*, Silicon Dioxide, Evening Primrose Oil, Modified Tapioca Starch, Potato Maltodextrin, Vitamin E (as D-Alpha Tocopheryl Acetate), *Lactobacillus salivarius*, Acacia Gum, Sucrose, Corn Starch, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, Vitamin A (as Retinyl Acetate) Antioxidants (Ascorbic Acid, Ascorbyl Palmitate & Natural Mixed Tocopherols), Corn Oil, Sunflower Oil DL-Alpha Tocopherol.

COMMERCIAL SENSITIVE INFORMATION STRICTLY FOR INTERNAL USE ONLY

FULL ALLERGEN DETAIL:

Added Yeast	No	GM Free	Yes	Dairy Free	No	Low Salt	-
Wheat Free	Yes	With Added Sugar	Yes	With Added Salt	No	No Salt	--
Gluten Free	Yes	No Added Sugar	-	Kosher	-	Halal	-

SUITABILITY:

Vegetarian	x	Vegan	x	Organic	x	Can Open capsule	x
Pregnancy	x	Breastfeeding	x	Children	x		

RECOMMENDED INTAKE:

Two teaspoons (approx. 5g) mixed well in water and taken daily with food or as professionally directed.

LABEL CAUTIONS/ADVISORY STATEMENTS:

If you are under medical supervision, please consult a doctor before use  
 This product contains vitamin A. Do not take if you are pregnant or if you are likely to become pregnant except on the advice of a doctor or ante-natal clinic  
 This product should not be used as a substitute for a varied and balanced diet and healthy lifestyle.  
 Do not exceed the stated recommended daily intake.  
 Do not purchase if the seal is broken.  
 Keep out of reach of children.  
 Refrigerate below 4oC and avoid direct sunlight and heat.

## Appendix F

### Marathon Des Sables Research Study

#### RACE GASTROINTESTINAL SYMPTOM QUESTIONNAIRE

Q: Did you stick to your pre-race nutritional strategy? If not, please describe what you changed.

Q: Please grade the quality of your sleep before each stage (0=no sleep; 3= low quality; 5 = medium quality (broken sleep); 7 = good quality/ unbroken; 10 = high quality/unbroken)

0	1	2	3	4	5	6	7	8	9	10

#### DURING EACH STAGE

*Please indicate whether the following apply to you*

(0=never or rarely; 1 = low severity; 2 = moderate severity; 3 = high severity)

Time taken =	0	1	2	3
Symptom				
1-General urge to urinate				
2-General urge to defecate				
3-Bloating severity				
4-Belching severity				
5-Flatulence severity				
6-Nausea				
7-Stomach/intestinal pain or discomfort				
8-Stomach/ intestinal cramping				
9-Headaches				
10-Dizziness				

*Roberts, J.D. & Roberts, M.G. (2013)*

Q: Did you think you suffered from any symptoms of endotoxemia? If yes, please grade the severity below (0=no evidence; 1= very mild; 3 = mild; 5 = moderate symptoms/ only part of either day; 7 = severe/ over full day 1 or 2; 10= very severe/ ill):

0	1	2	3	4	5	6	7	8	9	10



## Appendix G

### Baseline eHsp72 ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 3	Std 7	SM10	SM52	SM48	SM08	SM15	SM11	SM44	SM53	SF03
B	Blank	Std 3	Std 7	SM51	SM05	SM31	SM19	SF01	SF05	SM15	SM26	SM55
C	S0	Std 4	SM42	SM55	SM39	SM25	SF10	SM04	SM38	SM40	SM05	SM48
D	S0	Std 4	SM06	SF06	SM26	SM47	SF07	SM35	SM20	SM19	SM49	SM31
E	Std 1	Std 5	SM02	SM13	SF04	SM53	SM40	SM27	SM54	SM24	SF02	SM03
F	Std 1	Std 5	SM43	SF02	SM32	SM24	SM45	SM44	SM17	SM32	SF06	SM33
G	Std 2	Std 6	SM30	SM29	SM18	SM50	SM07	SM36	SM28	SM03	SM06	SF10
H	Std 2	Std 6	SF03	SM49	SM03	SM33	SF09	SF11	SM20	SM25	SM43	SM17

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.075	5.073	0.999	1.177	1.140	2.652	1.897	0.900	0.661	0.831	6.264	0.747
B	-0.098	5.024	1.001	0.681	0.941	0.884	1.480	0.886	0.660	0.955	0.748	1.945
C	0.777	2.926	0.644	1.906	0.786	1.267	2.116	0.970	0.945	9.448	0.895	2.735
D	0.707	3.097	0.771	1.075	0.982	0.968	0.597	1.021	1.361	1.562	0.898	0.752
E	12.773	1.897	0.587	2.824	0.590	5.992	8.186	4.373	1.005	1.176	1.806	0.761
F	13.484	1.899	0.511	1.629	0.878	1.067	0.807	0.891	1.249	1.062	1.157	2.708
G	8.040	1.212	1.797	0.937	1.992	0.708	0.994	0.726	0.801	0.718	1.052	2.210
H	8.199	1.335	0.647	0.809	0.524	2.318	0.912	1.291	1.520	1.167	0.840	1.127

### 7d pre-race eHsp72 ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 3	Std 7	SF03	SM11	SM33	SM35	SF07	SM42	SM11	SM35	SM42
B	Blank	Std 3	Std 7	SM28	SM32	SM54	SM52	SM47	SM31	SM30	SM15	SM07
C	S0	Std 4	SM31	SM55	SM30	SF09	SM15	SM26	SF11	SM06	SM18	SM40
D	S0	Std 4	SM07	SM49	SM51	SM48	SM24	SM08	SM39	SM38	SM50	SM13
E	Std 1	Std 5	SF11	SM45	SM06	SF02	SM18	SF04	SF03	SM33	SF07	SM28
F	Std 1	Std 5	SM40	SM53	SF10	SM04	SM05	SM27	SM55	SF09	SM26	SM49
G	Std 2	Std 6	SM39	SF05	SM38	SM02	SM50	SM10	SM45	SF02	SF04	SM53
H	Std 2	Std 6	SM13	SM44	SM17	SM36	SM19	SM03	SF05	SM02	SM10	SM44

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.067	4.358	0.958	0.829	0.725	2.690	1.022	0.598	0.693	0.761	1.112	0.658
B	-0.049	4.269	0.942	0.783	1.001	0.948	0.981	0.697	0.710	1.973	0.726	0.845
C	0.816	2.394	0.662	1.780	1.922	0.866	0.733	0.751	1.013	1.075	3.088	1.192
D	0.828	2.421	0.809	0.986	0.819	2.938	1.133	2.155	0.777	1.157	0.850	3.776
E	12.867	1.615	0.921	0.986	1.048	1.870	3.007	0.578	0.887	3.137	0.667	0.810
F	13.003	1.540	1.374	6.865	1.759	0.821	1.133	3.601	1.717	0.941	0.793	1.094
G	8.307	1.074	0.714	0.641	0.925	0.716	0.842	1.593	1.164	1.768	0.668	7.737
H	7.793	1.110	3.210	0.865	1.217	0.673	1.586	0.676	0.828	0.765	1.896	1.055

### 6-8h post race eHsp72 ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 3	Std 7	SM28	SM13	SM03	SF03	SM48	SM38	SM30	SM02	SM49
B	Blank	Std 3	Std 7	SM27	SM11	SM02	SF02	SM47	SM36	SM24	SF10	SM47
C	S0	Std 4	SM36	SM26	SM10	SF11	SM55	SM45	SM33	SM18	SM28	SM44
D	S0	Std 4	SM35	SM24	SM08	SF10	SM54	SM44	SM30	SM15	SF07	SM42
E	Std 1	Std 5	SM33	SM19	SM07	SF09	SM53	SM43	SM27	SM11	SF04	SM39
F	Std 1	Std 5	SM32	SM18	SM06	SF07	SM52	SM42	SM29	SM08	SF02	SM38
G	Std 2	Std 6	SM31	SM17	SM05	SF05	SM50	SM40	SF11	SM06	SM54	SM43
H	Std 2	Std 6	SM30	SM15	SM04	SF04	SM49	SM39	SF11	SM04	SM52	SF09

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.034	6.173	1.967	1.911	5.662	1.608	2.092	7.299	2.499	3.293	1.908	2.551
B	0.034	6.588	1.857	8.091	1.548	1.613	3.273	1.85	1.704	2.146	3.085	1.672
C	1.893	4.537	1.448	1.868	2.667	1.852	3.814	2.518	5.757	5.742	2.291	3.374
D	1.95	4.437	1.96	2.204	4.26	2.8	1.937	3.193	3.415	1.917	1.659	2.537
E	13.26	2.91	5.746	2.789	2.162	1.601	12.78	1.56	7.695	1.864	1.785	2.322
F	15.08	3.024	1.927	7.526	2.103	1.492	2.211	1.882	2.211	4.058	3.537	2.156
G	10.43	2.575	1.536	2.323	2.217	1.568	1.775	2.224	2.497	2.2	1.938	2.079
H	8.36	2.032	2.887	2.107	2.217	1.576	2.782	2.263	2.67	2.482	2.724	2.374

### 7d post-race eHsp72 ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Std 3	Std 7	SM11	SM45	SM39	SM47	SF02	SM54	SF04	SM30	SM08
B	Blank	Std 3	Std 7	SM40	SM48	SM10	SF05	SM42	SM17	SM50	SM02	SM18
C	S0	Std 4	SF02	SM54	SF04	SM30	SM08	SF10	SF09	SM33	SM19	SM26
D	S0	Std 4	SM42	SM17	SM50	SM02	SM18	SF07	SM06	SM32	SM28	SM24
E	Std 1	Std 5	SF10	SF09	SM33	SM19	SM26	SF03	SM53	SF11	SM13	SM55
F	Std 1	Std 5	SF07	SM06	SM32	SM28	SM24	SM35	SM52	SM27	SM05	SM43
G	Std 2	Std 6	SF03	SM53	SF11	SM13	SM43	SM11	SM45	SM39	SM47	SM47
H	Std 2	Std 6	SM35	SM52	SM27	SM05	SM55	SM40	SM48	SM10	SF05	SF03

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.048	4.320	1.050	0.744	1.087	0.916	0.748	1.809	1.018	0.754	2.068	2.278
B	-0.024	4.454	1.122	1.092	3.812	1.669	0.903	0.785	1.118	1.016	0.945	2.757
C	0.805	3.036	1.590	0.881	0.676	2.071	2.096	2.341	0.820	3.612	1.745	0.839
D	0.905	3.121	0.746	1.066	0.917	0.832	4.237	0.663	0.881	0.995	0.871	1.574
E	13.827	1.998	2.073	0.770	4.005	1.936	0.955	0.767	8.223	1.137	3.648	1.543
F	12.629	1.971	0.716	0.941	1.111	0.906	1.387	0.975	0.971	3.604	1.354	0.861
G	8.554	1.277	0.848	9.854	1.016	4.209	0.844	0.853	0.988	1.068	0.725	0.751
H	7.526	1.439	0.961	1.139	4.108	1.364	2.108	1.372	4.531	2.072	0.928	1.032

### Baseline LPS ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	A	A	B	B	C	C	D	D	E	E
B	F	F	SM24	SM24	SM26	SM26	SF07	SF07	SM33	SM33	SM38	SM38
C	SM31	SM31	SM18	SM18	SM10	SM10	SF03	SF03	SM30	SM30	SF02	SF02
D	SM35	SM35	SM06	SM06	SF09	SF09	SM55	SM55	SM03	SM03	SM36	SM36
E	SM39	SM39	SM27	SM27	SM49	SM49	SM07	SM07	SF05	SF05	SM54	SM54
F	SM28	SM28	SM50	SM50	SM15	SM15	SM45	SM45	SM43	SM43	SM17	SM17
G	SM05	SM05	SF11	SF11	SM13	SM13	SM52	SM52	SM48	SM48	SM40	SM40
H	SF10	SF10	SM32	SM32	SM44	SM44	SF04	SF04	SM53	SM53	SF06	SF06

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	A	A	B	B	C	C	D	D	E	E
B	F	F	6.761	6.850	5.782	6.405	6.939	10.052	5.871	10.319	8.540	9.074
C	13.433	13.611	14.500	14.500	10.319	10.141	8.006	8.006	11.832	8.718	11.654	11.654
D	11.120	11.476	7.028	7.473	12.632	14.678	11.387	11.298	16.458	18.237	5.338	4.181
E	16.013	17.525	10.230	14.234	7.473	7.473	13.255	11.921	15.212	16.013	11.565	9.074
F	13.789	13.789	13.789	13.967	9.875	9.608	13.611	13.878	8.718	7.028	6.939	7.206
G	9.964	9.163	-1.156	3.025	10.408	10.675	8.362	7.917	7.917	10.675	3.914	3.736
H	8.629	9.252	8.896	8.362	6.316	7.295	54.355	56.223	11.298	9.430	SF06	SF06

### Pre-race LPS ELISA plate layout

Pre-race	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	A	A	B	B	C	C	D	D	E	E
B	F	F	SM42	SM42	SM03	SM03	SM10	SM10	SM27	SM27	SF04	SF04
C	SM08	SM08	SM47	SM47	SM26	SM26	SF07	SF07	SM19	SM19	SM50	SM50
D	SM05	SM05	SM02	SM02	SM18	SM18	SM24	SM24	SM15	SM15	SM04	SM04
E	SF02	SF02	SM48	SM48	SF09	SF09	SM06	SM06	SF10	SF10	SM45	SM45
F	SM15	SM15	SM55	SM55	SM28	SM28	SF03	SF03	SM36	SM36	SF05	SF05
G	SM07	SM07	SM52	SM52	SM53	SM53	SM31	SM31	SM35	SM35	SM11	SM11
H	SM40	SM40	SM17	SM17	SM44	SM44	SF11	SF11	SM38	SM38	SM30	SM30

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	A	A	B	B	C	C	D	D	E	E
B	F	F	11.921	14.856	14.500	15.123	15.123	13.789	9.786	13.433	69.300	70.456
C	5.871	7.028	11.565	11.476	12.188	10.586	4.181	5.249	24.286	9.430	12.188	13.878
D	8.273	9.252	9.074	10.942	19.037	18.593	8.273	7.828	8.362	8.273	10.141	9.608
E	11.387	12.632	15.924	16.902	24.642	24.642	12.365	8.896	12.810	10.586	13.700	13.255
F	8.362	8.273	9.875	12.188	17.881	18.860	10.497	9.430	14.500	16.902	20.995	20.194
G	6.138	7.473	9.875	12.632	26.866	27.844	17.792	13.967	9.519	8.896	7.651	6.850
H	3.203	3.736	7.295	8.540	11.298	11.476	8.807	8.451	12.010	10.675	8.896	7.206

### 6-8h Post-race LPS ELISA plate layout

Post-race	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	SF02	SF02	SF03	SF03	SF04	SF04	SF05	SF05	SF07	SF07	SF09	SF09
<b>B</b>	SF10	SF10	SF11	SF11	SM02	SM02	SM03	SM03	SM04	SM04	SM05	SM05
<b>C</b>	SM06	SM06	SM07	SM07	SM08	SM08	SM10	SM10	SM11	SM11	SM13	SM13
<b>D</b>	SM15	SM15	SM17	SM17	SM18	SM18	SM19	SM19	SM24	SM24	SM26	SM26
<b>E</b>	SM27	SM27	SM28	SM28	SM30	SM30	SM31	SM31	SM32	SM32	SM33	SM33
<b>F</b>	SM35	SM35	SM36	SM36	SM38	SM38	SM39	SM39	SM40	SM40	SM42	SM42
<b>G</b>	SM43	SM43	SM44	SM44	SM45	SM45	SM47	SM47	SM48	SM48	SM49	SM49
<b>H</b>	SM50	SM50	SM52	SM52	SM53	SM53	SM54	SM54	SM55	SM55	Blank	Blank

Post-race	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	31.581	33.716	6.227	6.049	22.685	23.130	7.828	9.074	11.031	9.875	8.896	9.697
<b>B</b>	13.344	13.789	2.224	4.448	11.298	10.853	8.540	9.163	4.359	4.715	6.494	5.960
<b>C</b>	4.626	4.893	25.087	24.375	8.629	10.230	3.203	4.626	4.537	4.537	15.924	18.237
<b>D</b>	8.273	9.252	5.427	4.626	13.700	11.743	10.586	11.031	10.319	8.273	3.825	4.181
<b>E</b>	9.252	7.740	15.746	13.344	-2.669	6.138	6.227	5.160	8.718	7.384	0.623	2.580
<b>F</b>	2.669	2.580	1.512	1.690	4.804	5.427	7.740	7.117	11.209	8.273	11.654	7.473
<b>G</b>	23.219	19.660	12.632	12.010	17.703	16.280	3.825	1.868	3.114	2.758	5.160	5.604
<b>H</b>	6.227	5.427	18.682	19.660	8.540	8.896	6.850	4.982	18.771	19.927	Blank	Blank

**7d post-race LPS ELISA plate layout**

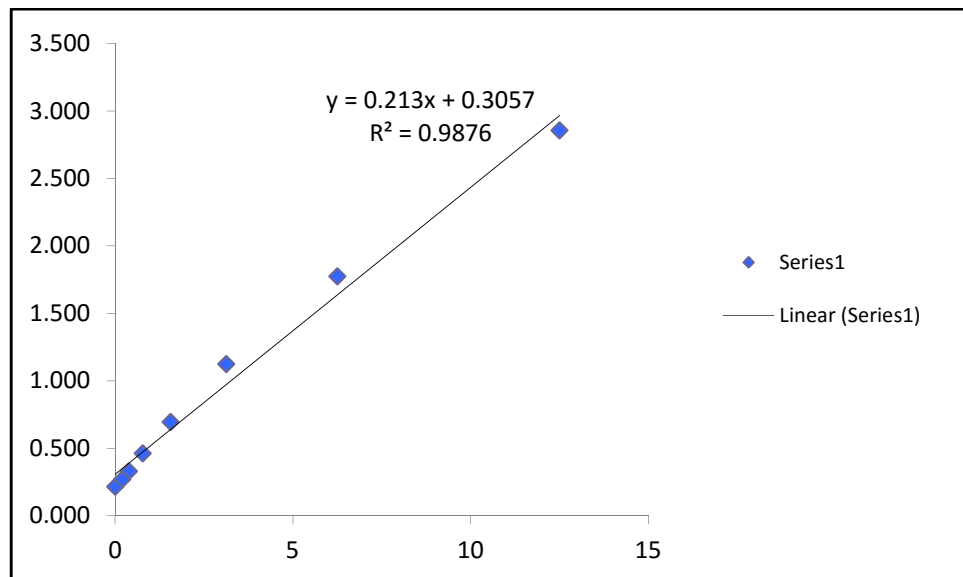
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	Blank	Blank	A	A	B	B	C	C	D	D	E	E
<b>B</b>	F	F	SF02	SF02	SM42	SM42	SF09	SF09	SM17	SM17	SM54	SM54
<b>C</b>	SM40	SM40	SM11	SM11	SM35	SM35	SF03	SF03	SF07	SF07	SF10	SF10
<b>D</b>	SM06	SM06	SM53	SM53	SM52	SM52	SM45	SM45	SM48	SM48	SF04	SF04
<b>E</b>	SM50	SM50	SM33	SM33	SM32	SM32	SM13	SM13	SM28	SM28	SM19	SM19
<b>F</b>	SM02	SM02	SM30	SM30	SM10	SM10	SM39	SM39	SM27	SM27	SF11	SF11
<b>G</b>	SM05	SM05	SM47	SM47	SF05	SF05	SM08	SM08	SM18	SM18	SM26	SM26
<b>H</b>	SM24	SM24	SM43	SM43	SM55	SM55	SF02	SF02	SF09	SF09	SM06	SM06

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	Blank	Blank	A	A	B	B	C	C	D	D	E	E
<b>B</b>	F	F	12.010	13.077	20.817	21.528	17.258	19.215	7.028	6.939	12.899	13.522
<b>C</b>	8.629	7.917	4.003	3.114	11.031	12.188	5.338	4.626	8.985	9.697	5.604	5.604
<b>D</b>	5.427	5.338	14.945	14.856	13.522	14.412	12.010	12.010	4.270	3.203	11.209	10.141
<b>E</b>	19.660	25.887	50.707	53.109	23.752	25.532	9.697	6.761	12.810	12.188	11.031	6.049
<b>F</b>	18.682	19.660	12.810	13.255	4.092	4.092	13.700	14.500	4.359	3.825	0.801	2.402
<b>G</b>	18.237	19.037	6.405	5.782	9.964	14.323	9.519	9.964	19.126	20.016	4.537	3.114
<b>H</b>	7.740	7.828	6.850	7.206	8.807	8.540	12.010	13.077	17.258	19.215	5.427	5.338



## Appendix H

### eHsp72 graph for linearity



## Appendix I

### LPS graph for linearity

